

Université de Sherbrooke

**ÉTUDE DES RÉPONSES IMMUNITAIRES INNÉES CHEZ LA VACHE LAITIÈRE ET
ANALYSE D'UN GÈNE CANDIDAT PROMETTEUR : L'OSTÉOPONTINE**

par

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Département de biochimie

Mémoire présenté à la faculté de médecine et des sciences de la santé

en vue de l'obtention du grade de

maître ès sciences (M.Sc.) en biochimie

5 janvier 2010

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RÉSUMÉ

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Chez le bovin laitier, la mammite est la maladie la plus importante pour l'économie agroalimentaire du secteur laitier et entraîne les plus grandes pertes pour le producteur. Depuis plusieurs décennies, les pressions génétiques visant l'amélioration des traits favorisant la production laitière, mesurée par l'augmentation du rendement (quantité et pourcentage) de lait, protéine et gras du lait, ont eu un effet délétère sur la santé de la vache. Prévenir cette maladie devient une priorité pour les producteurs. On vise à réduire l'usage d'antibiotiques et contenir l'émergence de pathogènes résistants. Afin de renverser la tendance actuelle, l'approche proposée est d'améliorer le système immunitaire des vaches par une sélection génétique favorable au renforcement de celui-ci.

L'objectif du projet consiste à identifier des gènes impliqués dans la réponse immunitaire innée de la vache laitière et qui contribueraient à développer une offensive face aux insultes pathogéniques causant la mammite. Un modèle d'infection *in vivo* a été créé par l'infusion d'*Escherichia coli* dans des quartiers de glandes mammaires de vaches saines. Des banques de gènes transcrits ont été créées à partir de l'ARNm des cellules du lait récoltées en début d'infection (cinq heures suivant l'introduction des pathogènes). L'analyse des banques a révélé la présence d'un gène connu pour activer les lymphocytes T, l'ostéopontine (*SPP1*). L'induction de ce gène fut confirmée lors d'essais d'infection *ex vivo* avec *Escherichia coli* sur des cellules sanguines de vaches saines. Comme *SPP1* est une cytokine produite tôt par les macrophages lors d'une infection bactérienne, elle pourrait avoir un impact important sur les défenses contre la mammite.

Afin d'identifier des variants génétiques de *SPP1*, la recherche de polymorphismes de nucléotides fut réalisée en comparant les séquences d'ADN génomique de deux groupes de taureaux de race Holstein présentant des valeurs d'élevage estimées (VÉE) extrêmes selon une cote attribuée aux cellules somatiques (CCS) du lait. Le séquençage de la région promotrice et des sept exons du gène a permis d'identifier quatre polymorphismes d'un seul nucléotide (SNP), soit *SPP1c.-1301G>A*, *SPP1c.-1251C>T*, *SPP1c.-430G>A* et *SPP1c.*41A>C*. Pour confirmer l'association de ces SNP dans la population Holstein, un plus grand nombre de taureaux furent génotypés et les haplotypes, déterminés. L'analyse statistique de l'association des VÉE pour la CCS à différentes lactations avec les quatre SNP et haplotypes présents dans la population a confirmé leur impact sur la CCS. L'étude a également porté sur les VÉE de certains autres critères de performance (e.g. pourcentages de gras et protéines du lait) qui seront aussi utiles aux producteurs laitiers afin d'évaluer l'impact de ces SNP sur la production laitière.

Certains SNP et leur localisation (promoteur, région 3' UTR) potentialisent l'impact génétique sur la VÉE pour la CCS. Comme cette dernière est en lien avec la mammite, certains SNP pourraient influencer la résistance à cette maladie. Aussi, les SNP découverts dans la région promotrice de *SPP1* sont localisés au site de reconnaissance de certains facteurs de transcription. Ils pourraient avoir un impact sur la liaison de ceux-ci à leur séquence consensus; ceci amènerait des effets sur la différenciation des lymphocytes T vers les voies de l'immunité cellulaire (Th1) et acquise (Th2). De cette manière, la réponse immunitaire serait modifiée et *SPP1* pourrait effectivement influencer la résistance à la mammite.

Mots clés : cote de cellules somatiques / immunité innée / mammite / ostéopontine /
polymorphisme d'un seul nucléotide de l'ADN

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LISTE DES ABBRÉVIATIONS

3' UTR : région 3' non traduite (*3' untranslated region*)

5' UTR : région 5' non traduite (*5' untranslated region*)

ACTB : beta actine (*actin, beta*)

ADN : acide désoxyribonucléique

ADNc : ADN complémentaire

ARN : acide ribonucléique

ARNm : ARN messenger

BLAST : Basic Local Alignment Search Tool

bp : paires de bases (*base pairs*)

BSA : albumine de sérum bovin (*bovine serum albumin*)

BTA : *Bos taurus* autosome

CCS : cote (ou compte) de cellules somatiques

CDN : réseau laitier canadien (*Canadian Dairy Network*)

cDNA : complementary DNA

CFU : unité formatrice de colonie (*colony forming unit*)

CMH II : complexe majeur d'histocompatibilité de classe II

DNA : deoxyribonucleic acid

dNTP : désoxynucléotide triphosphate (*deoxyribonucleotide triphosphate*)

EBV : estimated breeding value

E. coli : *Escherichia coli*

EDTA : acide éthylène diamine tetra acétique (*ethylenediaminetetraacetic acid*)

ELISA : enzyme-linked immunosorbent assay

Eta-1: early T lymphocyte activation-1

FBS : sérum fœtal bovin (*fetal bovine serum*)

g : unité de gravité (*unit of gravity*)

GAPDH : glyceraldéhyde-3-phosphate déshydrogénase (*glyceraldehyde-3-phosphate dehydrogenase*)

gDNA : ADN génomique (*genomic DNA*)

GLYCAM : molécule d'adhésion cellulaire dépendant de la glycosylation-1 (*glycosylation-dependent cell adhesion molecule 1*)

HBSS : Solution saline tamponnée HANKS (*Hank's buffered salt solution*)

IFN γ : interféron gamma (*interferon gamma*)

IKZF2 : Ikaros family zinc finger 2

IL : interleukine (*interleukin*)

INDEL : insertion et délétion (*insertion and deletion*)

ISRE : élément de réponse à la stimulation IFN (*interfeon-stimulated response element*)

LA : acrylamide linéaire (*linear acrylamide*)

mg : milligramme (*milligram*)

mL : millilitre (*milliliter*)

mM : millimolaire (*millimolar*)

mRNA : messenger RNA

NCBI : National Center for Biotechnology Information

NFAT : facteur nucléaire des lymphocytes T activés (*nuclear factor of activated T-cells*)

ng : nanogramme (*nanogram*)

nm : nanomètre (*nanometer*)

nM : nanomolaire (*nanomolar*)

nt : nucléotide (*nucleotide*)

OD : densité optique (*optical density*)

OPN : ostéopontine (*osteopontin*)

P : valeur de p (*p value*)

PAMP : motifs moléculaires associés aux pathogènes (*pathogen-associated molecular pattern*)

PBMC : cellules mononuclées du sang périphérique (*peripheral blood mononuclear cell*)

PBS : tampon phosphate salin (*phosphate buffered saline*)

PCR : réaction en chaîne par polymérase (*polymerase chain reaction*)

PFS : solution saline apyrogène (*pyrogen-free saline*)

PPIA : peptidylprolyl isomérase A (*peptidylprolyl isomerase A*)

qPCR : PCR quantitatif (*quantitative PCR*)

RNA : ribonucleic acid

RT : transcription inverse (*reverse transcription*)

SAS : Statistical Analysis System

S. aureus : *Staphylococcus aureus*

SCC : somatic cell count

SCS : somatic cell score

SCS1 : somatic cell score at first lactation

SCS2 : somatic cell score at second lactation

SCS3 : somatic cell score at third lactation

SNP : polymorphisme d'un seul nucléotide (*single nucleotide polymorphism*)

SP1 : facteur de transcription Sp1 (*Sp1 transcription factor*)

SPP1 : phosphoprotéine 1 sécrétée (*secreted phosphoprotein 1*)

SSH : hybridation soustractive suppressive (*suppressive subtractive hybridization*)

Tetra-primer ARMS-PCR : tetra-primer amplification refractory mutation system PCR

TF : facteur de transcription (*transcription factor*)

Th1 : lymphocyte T auxiliaire de type 1 (*T helper 1*)

Th2 : lymphocyte T auxiliaire de type 2 (*T helper 2*)

TLR : récepteur de type Toll (*Toll-like receptor*)

TNF α : facteur de nécrose tumorale (*tumor necrosis factor alpha*)

TSA : gélose tryptique de soja (*tryptic soy agar*)

TSB : bouillon tryptique de soja (*tryptic soy broth*)

U : unité d'enzyme (*enzyme unit*)

μ g : microgramme (*microgram*)

μ L : microlitre (*microliter*)

μ M : micromolaire (*micromolar*)

VÉE : valeur d'élevage estimée

INTRODUCTION

Ce projet fut élaboré pour répondre à une problématique, soit l'incidence élevée de la mammites chez les vaches laitières. Avec la sélection d'animaux offrant de meilleurs rendements en lait, la pression génétique a également favorisé la sélection d'animaux plus susceptibles aux infections de la glande mammaire (RAINARD et RIOLLET, 2006). Les mammites coûtent cher aux producteurs; elles affectent chaque année en moyenne deux vaches sur cinq (BRADLEY, 2002) et coûtent environ 180\$ par cas clinique (BAR *et al.*, 2008). Ces pertes sont reliées non seulement à la diminution de production de lait, mais aussi aux changements dans la composition du lait, aux coûts de remplacement dus à la réforme prématurée des vaches, aux coûts en traitements (soins vétérinaires, antibiotiques) sans compter la charge de travail additionnelle à la ferme. Or, les producteurs laitiers sont grandement intéressés par des animaux plus résistants à cette maladie. Cette tendance est encouragée par les consommateurs, beaucoup plus conscients aux dangers que comporte l'utilisation d'antibiotique, qui veulent une nourriture saine et sécuritaire (RUPP *et al.*, 2007). Il existe déjà certaines voies envisageables pour contrer la maladie, par exemple la vaccination des vaches contre les agents pathogènes, mais cette dernière n'est toujours pas au point (RAINARD et RIOLLET, 2006; RUPP *et al.*, 2009). Pour renverser cette tendance génétique à favoriser des animaux productifs au détriment de la santé de l'animal, l'étude des mécanismes de défenses naturelles des vaches contre les pathogènes responsables de ce fléau a été proposée. En effet, une meilleure connaissance des gènes qui peuvent potentiellement conférer une résistance s'avère essentielle. L'approche que nous proposons vise à identifier les variants génétiques favorables et augmenter leur fréquence dans la

population bovine par une sélection génétique d'animaux naturellement plus résistants à la mammite (KADARMIDEEN *et al.*, 2006; SORDILLO *et al.*, 1997). Comme les gènes reliés aux réponses immunitaires innées sont des cibles de choix pour la sélection génétique, le but du travail se résume à identifier des gènes, et leurs variants génétiques associés, impliqués dans la première ligne de défense. Il sera ensuite possible, de proposer certains allèles qui orienteront le choix des géniteurs mâles pour disséminer les allèles favorables dans la population et ainsi améliorer la résistance des vaches aux infections bactériennes causant la mammite. Conséquemment, on pourrait réduire l'utilisation d'antibiotiques et possiblement contenir l'émergence de pathogènes résistants. Tout en soutenant une production laitière élevée, on assisterait à une augmentation des revenus pour les producteurs laitiers puisque les coûts et les pertes reliés à cette maladie seraient réduits.

La présente recherche porte donc, en premier lieu, sur l'identification de gènes impliqués dans la première ligne de défense lors d'une mammite, créée dans un modèle d'infection *in vivo* par l'infusion d'*E. coli* dans des quartiers de glandes mammaires de vaches saines.

Le nombre de bactéries nécessaires pour l'introduction d'une mammite a préalablement été établie (100 CFU). Suite à l'injection dans certains quartiers de glande mammaire de vaches saines, le lait fut récolté et les cellules furent isolées pour extraire l'ARN présent. L'analyse des gènes transcrits fut réalisée à l'aide de l'hybridation suppressive et soustractive. L'ARN des cellules non-infectées est soustrait à celui des cellules infectées par une technique dérivée de la PCR favorisant l'amplification des gènes plus abondants.

Cette technique permet aussi d'éliminer les gènes qui sont constitutivement exprimés et faire ressortir les gènes transcrits présents dans la population de cellules infectées. À l'aide de cette technique, le gène de l'ostéopontine a été identifié.

En second lieu, suivant l'identification de l'ostéopontine comme étant induite en début d'infection bactérienne par *E.coli* dans la glande mammaire, l'étude porte sur la recherche de polymorphismes de l'ADN dans la séquence génomique de ce gène. Pour ce faire, deux populations de taureaux ayant des valeurs extrêmes pour le CCS (haut et bas) ont été comparées. Différentes portions du gène (la région promotrice et les sept exons) ont d'abord été amplifiées par PCR pour finalement être séquencées. Nous avons utilisé un assemblage (pool) d'ADN d'individus regroupés par phénotype pour le séquençage des régions génomiques. En comparant les deux groupes comportant un phénotype extrême, nous avons identifié des polymorphismes dans l'ADN. Cette technique s'est avérée efficace car un nouveau polymorphisme d'un seul nucléotide a été mis en lumière en plus d'identifier certains polymorphismes déjà rapportés dans la littérature. L'analyse des polymorphismes au sein 26 familles de taureaux a permis la construction des haplotypes et de déterminer leur fréquence respective dans la population de taureaux de race Holstein. Les études d'association des quatre polymorphismes de l'ADN identifiés et leurs haplotypes associés ont été réalisées avec les valeurs estimées d'élevage d'une plus grande population de taureaux. Des liens directs furent établis avec la cote de cellules somatiques (CCS) des taureaux, qui est un phénotype inféré suivant l'analyse des performances de sa progéniture, disponible pour chaque individu via le Réseau laitier canadien (CDN; www.cdn.ca). La CCS représente un indice (le seul) de santé de la

glande mammaire (CASTILLO-JUAREZ *et al.*, 2002; HERINGSTAD *et al.*, 2008; RUPP *et al.*, 2009). Des études d'association ont aussi été réalisées sur d'autres traits de production laitière pour vérifier si les SNP ont des effets sur celle-ci.

La mammite est une maladie bien caractérisée. Cette inflammation de la glande mammaire est principalement causée par une infection bactérienne. Elle est habituellement classifiée selon l'agent pathogène qui en est responsable et se divise en deux principales catégories : la mammite environnementale – *E. coli* est le principal agent responsable – et chronique, une mammite dite contagieuse occasionnée par la transmission horizontale de pathogènes Gram-positifs. Bien quelle soit très fulgurante, la mammite environnementale se résorbe habituellement en quelques jours (LEE *et al.*, 2006). Elle se caractérise par des signes cliniques apparents tels que l'inflammation des quartiers infectés, des grumeaux dans le lait et l'état général de la vache qui se détériore (fièvre, perte d'appétit) (PETZL *et al.*, 2008). Cette infection peut occasionner une perte de quartiers ayant pour conséquence la réforme précoce de l'animal et, dans des cas plus extrêmes, la mort de l'animal (BURVENICH *et al.*, 2003). Par opposition à la mammite environnementale, les symptômes de la mammite contagieuse sont plus variables et les signes cliniques (e.g. fièvre) sont parfois notables, parfois discrets, voire innapercus. *Staphylococcus aureus* (*S. aureus*) est l'agent responsable des mammites contagieuses. Il se transmet entre les animaux lors de la traite alors que les barrières naturelles du pis sont moins étanches (BRADLEY, 2002), les techniques de récolte du producteur n'étant pas tout à fait adéquates. La mammite contagieuse entraîne des pertes économiques importantes et une CCS élevée. La production de lait baisse et la capacité de la glande

mammaire diminue graduellement puisque cette maladie peut persister plusieurs mois, voire toute la vie de l'animal (BANNERMAN *et al.*, 2004).

Le compte des cellules somatiques (CCS) dans le lait est répandu comme moyen de détection de la mammite contagieuse; ce compte peut cependant fluctuer énormément et amener les producteurs à penser que l'animal infecté est guéri alors qu'il n'en est rien. En effet, certains pathogènes peuvent se cacher périodiquement dans les cellules épithéliales. Dans une glande mammaire en santé, les principales cellules présentes sont les macrophages (SORDILLO et STREICHER, 2002). Lors d'une infection bactérienne, ils relâchent des molécules responsables du chimiotactisme des neutrophiles qui aura pour effet l'augmentation considérable du compte des cellules somatiques du lait (passant facilement de 100 000 à 1 000 000 cellules/mL (SORDILLO *et al.*, 1997)). Les neutrophiles représentent alors 90% des cellules dans une glande mammaire infectée (LAHOUESSA *et al.*, 2007).

La distribution du compte de cellules somatiques n'est pas uniforme au sein d'une population. Elle est généralement convertie en une valeur logarithmique (RUPP et BOICHARD, 2003) que l'on appelle alors cote de cellules somatiques (CCS). Les taureaux reçoivent une VÉE sur les cotes, calculée pour chaque lactation, en utilisant les données de toute sa descendance. Une cote de 3.00 est la valeur ajustée pour la moyenne de chaque race. Une cote inférieure à 3 est favorisée par rapport à une cote élevée. Malgré tous les efforts déployés en prévention et en traitement afin de diminuer l'incidence de la mammite, elle demeure la maladie la plus importante chez la vache laitière (BRADLEY,

2002). Il y a donc un besoin urgent d'agir et d'améliorer les défenses naturelles des animaux. La sélection génétique, en l'occurrence, est une technique de choix.

On constate qu'en sélectionnant pour des animaux avec une CCS faible, les producteurs laitiers diminuent l'incidence de mammites dans leur troupeau (LAHOUASSA *et al.*, 2007). En ciblant les variants génétiques favorables de certains facteurs clés du système immunitaire, on envisage l'amélioration de la santé de l'animal par la sélection génétique. Certaines études d'association entre des polymorphismes d'un seul nucléotide dans un gène relié à l'immunité et la CCS furent réalisées dans le but d'identifier des gènes intéressants pour la sélection génétique d'animaux plus résistants à la mammité (LEYVA-BACA *et al.*, 2008; LEYVA-BACA *et al.*, 2007; PANT *et al.*, 2007; SHARMA *et al.*, 2006; YOUNGERMAN *et al.*, 2004). Par exemple, les auteurs de ces études ont identifié des polymorphismes de l'ADN dans les gènes des chimiokines IL-8 et CCL2 et de récepteurs à chimiokines CXCR1, CXCR2 et IL8RA. Ces derniers sont reconnus comme étant des médiateurs essentiels pour la communication des cellules immunitaires lors des processus inflammatoires. D'autres études visent plutôt les gènes des récepteurs cellulaires CARD15 et TOLL-4, impliqués dans la reconnaissance des pathogènes et dans l'initiation des réponses inflammatoires. Nous avons réalisé une étude similaire, en utilisant cependant un candidat fonctionnel, soit un gène identifié dans un modèle d'infection *in vivo* chez la vache laitière. Ceci est d'autant plus intéressant puisque le lien direct de ce gène de l'immunité innée avec la mammité se confirme.

L'immunité innée est un mécanisme commun entre les espèces, car très bien conservée au cours de l'évolution (NAKAJIMA *et al.*, 2008). L'immunité innée est considérée comme étant la première ligne de défense de l'organisme contre les agents pathogènes, faisant appel à des mécanismes de reconnaissance assez primitifs. À la surface des cellules immunitaires phagocytaires de la glande mammaire – en majorité des macrophages dans une glande mammaire en santé (SOHN *et al.*, 2007) et neutrophiles – se trouvent des récepteurs, non spécifiques à l'agent pathogène, mais bien à des molécules très conservées à leur surface. Ces molécules, telles des lipopolysaccharides (bactéries Gram-négatives) ou acides lipotéichoïques (bactéries Gram-positives), sont reconnues par les cellules qui pourront phagocyter les pathogènes. La présentation des antigènes aux lymphocytes T se fait ensuite via le complexe majeur d'histocompatibilité de classe II (CMH II) à la surface des cellules phagocytaires. Par la suite, les lymphocytes T pourront se différencier en cellules T auxiliaires (helper en anglais) de type 1 (Th1) et participer aux réactions inflammatoires en interagissant avec les cellules phagocytaires afin de les aider à détruire les pathogènes. Il existe aussi les lymphocytes T de type Th2; ceux-ci sécrètent plutôt des cytokines anti-inflammatoires, comme l'interleukine (IL)-10, et favorisent l'immunité humorale, c.-à-d. la production d'immunoglobulines par les lymphocytes B et l'établissement de l'immunité acquise (NAGAI *et al.*, 2001). La sécrétion de cytokines est au cœur de la communication cellulaire permettant d'assurer les différentes fonctions du système immunitaire. Ces protéines régulent différents mécanismes dont l'apoptose, l'inflammation, la prolifération des lymphocytes B et T ainsi que leur différenciation, le recrutement des leucocytes au site d'inflammation et l'activité des phagocytes (ALLUWAIMI, 2004; RIOLLET *et al.*, 2000). Dans les réactions

précoces de l'immunité innée, IL-1 et IL-8 vont permettre la chimioattraction des neutrophiles et macrophages au site d'infection (WANG et DENHARDT, 2008). IL-12 permet la différenciation des lymphocytes T activés en cellules Th1 (ALLUWAIMI, 2004) et contribue à l'immunité innée via la sécrétion d'IFN γ , un activateur des neutrophiles et des macrophages. Comme elles occupent un rôle central dans les défenses de l'hôte, les cytokines sont visées comme cibles thérapeutiques et solutions possibles pour l'amélioration du système de défense contre les maladies comme la mammites (ALLUWAIMI, 2004; FINLAY et HANCOCK, 2004; RAINARD et RIOLLET, 2006; SORDILLO *et al.*, 1997).

L'ostéopontine, une cytokine importante de l'immunité innée, est une protéine complexe aux fonctions et aux modifications post-traductionnelles multiples, ce qui lui a valu plusieurs appellations. Elle fut d'abord appelée ostéopontine (OPN, terme le plus couramment utilisé) suite à sa découverte dans le cortex osseux (HERRING et KENT, 1963). Elle est aussi considérée comme étant une cytokine activatrice des lymphocytes T et de l'immunité cellulaire (O'REGAN *et al.*, 2000), d'où son nom Eta-1 (pour Early T lymphocyte activation-1). Finalement, phosphoprotéine 1 sécrétée (SPP1) est son nom officiel établi selon qu'elle se retrouve sécrétée dans tous les fluides biologiques de l'organisme (DENHARDT et NODA, 1998; WEBER et CANTOR, 1996) dont le lait (SORENSEN et PETERSEN, 1993). Le projet présenté dans ce mémoire porte essentiellement sur les rôles de l'ostéopontine comme cytokine produite par les macrophages et les lymphocytes T activés (PATARCA *et al.*, 1993; SODEK *et al.*, 2000) lors d'une infection bactérienne, et de molécule modulatrice de l'immunité cellulaire.

L'ostéopontine régule entre autres l'expression des cytokines IL-12 et IL-10 par les macrophages (ASHKAR *et al.*, 2000; HUMMELSHOJ *et al.*, 2006). En effet, une souris *Spp1*^{-/-} produit une moins grande quantité d'IL-12 et IFN γ , tandis que la production d'IL-10 se voit augmentée, ce qui modifie l'immunité cellulaire et procure de moins bonnes défenses contre les infections virales et bactériennes (ASHKAR *et al.*, 2000). Comme SPP1 est sécrétée abondamment par les macrophages lors d'une infection bactérienne, cette cytokine pourra assurer la régulation des diverses fonctions des macrophages, dont la sécrétion d'IL-12, tel que mentionné ci-dessus, qui favorisera la différenciation des lymphocytes T activés en cellules Th1 (PATARCA *et al.*, 1993; WANG et DENHARDT, 2008; WEBER et CANTOR, 1996). Ces cellules T pourront à leur tour stimuler l'immunité cellulaire et la destruction des pathogènes intracellulaires via la production de IFN γ , TNF α et IL-2 (QUIRION *et al.*, 2009), cytokines qui stimulent l'activité des cellules phagocytaires. D'un autre côté, *SPP1* inhibe la production d'IL-10, une cytokine anti-inflammatoire de la voie Th2 (immunité humorale, production d'anticorps), ce qui favorise la voie Th1 (WANG et DENHARDT, 2008). Pour ces raisons, on attribue à l'ostéopontine le rôle de médiateur entre la voie Th1 et Th2. Ses rôles ne s'arrêtent pas à l'immunité seulement. En effet, l'ostéopontine est produite par les reins et sécrétée dans l'urine où elle empêche la précipitation du calcium (KHAN et CANALES, 2009). Elle est aussi essentielle au remodelage de l'os mature (DENHARDT *et al.*, 2001). Cette protéine est souvent élevée dans plusieurs cas de cancer (cancer du poumon et cancer du sein par exemple) et est beaucoup étudiée comme cible thérapeutique (JOHNSTON *et al.*, 2008).

Beaucoup d'études ont porté sur l'importance de *SPP1* dans les réponses immunitaires (ASHKAR *et al.*, 2000; CHABAS, 2005; DENHARDT *et al.*, 2001; KONNO *et al.*, 2006). D'autres études ont porté sur l'association de *SPP1* (c.-à-d. polymorphismes d'un seul nucléotide) avec certaines maladies, notamment les maladies autoimmunes (AGNHOLT *et al.*, 2007; CHIOCCHETTI *et al.*, 2004; FORTON *et al.*, 2002) et le cancer (RANGASWAMI *et al.*, 2006; WEBER, 2001). Finalement, chez les vaches laitières, il existe des études d'associations entre certains polymorphismes de l'ADN de *SPP1* et des traits de production laitière (ALLAN *et al.*, 2007; KHATIB *et al.*, 2007; LEONARD *et al.*, 2005; SCHNABEL *et al.*, 2005; WHITE *et al.*, 2007). Malgré toutes ces recherches, aucune ne fait de lien entre l'ostéopontine et la mammite (via le CCS). C'est pourquoi nous nous sommes penchés sur le potentiel génétique de cette cytokine en analysant l'association possible entre des polymorphismes de l'ADN dans *SPP1* et la CCS, qui est un indice de mammite (HERINGSTAD *et al.*, 2008). Par l'identification d'allèles favorisant la résistance à la mammite et qui, par conséquent, diminuent le CCS, il sera possible de les valoriser dans la population bovine canadienne de race Hosltein lors de la reproduction en sélectionnant des bêtes avec de plus grandes résistances naturelles aux infections de la glande mammaire.

Les objectifs de ce projet étaient donc d'identifier des gènes exprimés rapidement lors d'une mammite. Ensuite, de trouver des polymorphismes dans la séquence génomique des gènes et de voir s'ils ont un effet sur le CCS surtout, mais aussi sur la production laitière. À plus long terme, les objectifs sont de confirmer ou d'infirmer la liaison de certains facteurs de transcription sur la séquence génomique de l'ostéopontin, de vérifier de

manière *in vivo* les effets qu'ont ces polymorphismes lorsqu'une vache est confrontée à une infection. Ultimement, ce serait de sélectionner dans la population bovine les allèles favorables à la défense contre la mammite.

AVANT-PROPOS DE L'ARTICLE

L'article ci-joint au mémoire s'intitule « Osteopontin: an early innate immune marker of *Escherichia coli* mastitis harbors genetic polymorphisms with possible links with resistance to mastitis». Les auteurs sont Niel A Karrow, Catherine Thibault, Jessika St-Pierre, Martin Lessard, Nathalie Bissonnette ainsi que moi-même, Karin Alain. À cet avant-propos sont joints les formulaires d'autorisation d'intégration d'un article écrit en collaboration à un mémoire dûment signés par chacun des coauteurs. L'article a été soumis à la revue BMC Genomics le 30 avril 2009. Depuis ce temps, l'article a été accepté pour publication le 18 septembre 2009 et la référence est « Alain K, Karrow NA, Thibault C, St-Pierre J, Lessard M, Bissonnette N: **Osteopontin: an early innate immune marker of *Escherichia coli* mastitis harbors genetic polymorphisms with possible links with resistance to mastitis.** BMC Genomics 2009, **10**:444 ».

L'autorisation d'intégration d'un article écrit en
collaboration à un mémoire ou une thèse a été retirée à
cause des renseignements personnels

RÉSUMÉ DE L'ARTICLE

L'article qui suit porte sur l'étude des gènes impliqués dans l'immunité innée lors de cas de mammites chez les vaches laitières. La mammite est la maladie la plus importante chez la vache laitière et demeure la principale cause de pertes économiques pour le producteur. L'identification de gènes impliqués dans l'amélioration de la réponse immunitaire innée et de leurs variants génétiques est donc essentielle pour faciliter les défenses face à cette maladie. En utilisant ces polymorphismes de l'ADN comme marqueurs pour la sélection de taureaux géniteurs, la descendance de ces animaux pourrait bénéficier d'une résistance accrue aux infections, permettant ainsi de réduire l'utilisation d'antibiotiques. Suite à l'identification d'un gène clé impliqué dans la réponse immunitaire, nous avons évalué son potentiel génétique pour la sélection d'animaux plus résistants naturellement à l'infection de la glande mammaire.

L'ostéopontine fut mise en évidence par la technique d'hybridation suppressive et soustractive sur des banques d'ADNc de cellules du lait de vaches soumises à une infection à *Escherichia coli*. L'amplification de ce gène en début d'infection dans des modèles tant *in vivo* qu'*ex vivo* a été investiguée par PCR quantitatif (qPCR). La recherche de polymorphismes de l'ADN pour ce gène *SPP1* fut réalisée sur deux groupes de taureaux présentant des valeurs d'élevage estimées (VÉE) extrêmes pour la cote de cellules somatiques (CCS), un indice de santé de la glande mammaire. Les amplicons de la région promotrice (5' UTR) et des sept exons du gène ont été générés et ont permis l'identification de quatre polymorphismes d'un seul nucléotide : *SPP1c.-1301G>A*, *SPP1c.-1251C>T*, *SPP1c.-430G>A* et *SPP1c.*41A>C*. Une population comprenant 578

taureaux de race Holstein, répartis en 26 familles différentes fut génotypée et les haplotypes déterminés. Ce groupe présentait une moyenne de la CCS de 3.01, représentative de la moyenne de la race (i.e. 3.00). Les études statistiques réalisées démontrent que le polymorphisme *-1301G>A* a un impact sur la valeur de la CCS ($P < 0.001$), ce qui n'a pas été montré par substitution allélique. Le polymorphisme **41A>C* influe sur le CCS ($P < 0.001$). Cet effet est aussi observé par substitution allélique ($P < 0.05$), tout comme pour les SNP *-1251C>T* et *-430G>A*. Les analyses statistiques ont également démontré les différences entre les haplotypes pour les VÉE pour la CCS à la 1^{re} ($P = 0.012$), 2^e ($P < 0.001$) et 3^e ($P < 0.001$) lactation ainsi que pour la lactation globale ($P < 0.001$). Pour ce qui est des traits de production, les analyses d'effet moyen de substitution allélique indiquent que le polymorphisme *-1301G>A* a un impact sur le rendement en lait pour la 2^e ($P = 0.027$) et la 3^e ($P = 0.046$) lactation, sur le pourcentage de gras pour la 1^{re} ($P = 0.004$), 2^e ($P = 0.012$) et la 3^e ($P = 0.009$) lactation et sur le pourcentage de protéines pour la 2^e ($P = 0.030$) et la 3^e ($P = 0.036$) lactation. Il existe aussi des différences statistiques entre les haplotypes pour le rendement en gras ($P = 0.044$) et pour le pourcentage de gras ($P = 0.043$).

Cet article montre l'association entre les polymorphismes d'un seul nucléotide dans le gène de *SPP1* et l'effet sur les cellules somatiques du lait. La susceptibilité potentielle à la mammite a aussi été évaluée. La liaison probable de certains facteurs de transcription (SP1 et IKAROS) dans la région promotrice ou l'absence de de liaison en raison de la présence du polymorphisme (de nucléotide), semblent au cœur de cet effet. Effectivement, l'emplacement de ces polymorphismes d'un seul nucléotide aux sites de

reconnaissance de certains facteurs de transcription pourrait réguler les processus d'immunité de type Th1 et Th2. Certains allèles de l'ostéopontine pourraient devenir éventuellement des cibles de choix ou des marqueurs importants lors de la sélection génétique contre la mammite et autres infections.

Pour ce qui est de ma contribution, j'ai rédigé en grande partie la première version du manuscrit comprenant les sections matériel et méthode, résultats, introduction, discussion et conclusion. J'ai contribué à la conception de la figure 1 et élaboré la figure 2 ainsi que les tableaux 1 à 8. Le travail qui y représenté, soit la construction de la librairie d'ADNc, l'extraction d'ADN génomique, le séquençage, la recherche de polymorphismes de l'ADN dans le gène de l'ostéopontine chez certains individus et le génotypage d'une plus grande population (578 taureaux) sont le produit de mon travail. Catherine Thibault et Jessika St-Pierre, une autre étudiante à la maîtrise sous la direction de Nathalie Bissonnette, ont élaboré les essais d'infection sur les vaches. L'équipe du Dr Lessard ont réalisé les essais de cytométrie de flux. L'équipe du Dr Karrow a fournis l'ADN de plus de 500 taureaux et m'a beaucoup aidé lors de l'utilisation de l'algorithme HAPROB. Finalement, c'est le Dr Bissonnette qui a élaboré ce projet, proposé les expériences, établi les contacts avec les autres équipes, rédigé une partie de l'article, corrigé la première version, soumis et assuré la publication de l'article.

L'ARTICLE

Osteopontin: an early innate immune marker of *Escherichia coli* mastitis harbors genetic polymorphisms with possible links with resistance to mastitis

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ABSTRACT

Background

Mastitis is the most important disease in dairy cows and it causes significant lost of profit to producers. Identification of the genes, and their variants, involved in innate immune responses is essential for the understanding of this inflammatory disease and to identify potential genetic markers for resistance to mastitis. The progeny of dairy cows would benefit from receiving favourable alleles that support greater resistance to infection, thus reducing antibiotic use. This study aims to identify a key gene in the innate immune response to mastitis, led us to evaluate its genetic association with somatic cell score (SCS), which is an indicator of clinical mastitis, and to evaluate its impact on other traits related to milk production.

Results

The osteopontin transcript (*SPP1*) was identified in the somatic cells from cows experimentally infected with *Escherichia coli*. By selecting bulls with extreme estimated breeding values (EBVs) for SCS, which is an indicator of mammary gland health, four DNA polymorphisms in the *SPP1* genomic sequence were found. Statistical analysis revealed that the SNP *SPP1c.-1301G>A* has an impact on EBV for SCS ($p < 0.001$) Using an allele substitution model, *SPP1c.-1251C>T*, *SPP1c.-430G>A*, and *SPP1c.*40A>C* have an impact on SCS whereas *SPP1c.-1301G>A* has an effect on the EBVs for milk yield (second and third lactations), fat and protein percentages (all three lactations). Analysis revealed statistically significant differences between haplotype

groups at a comparison-wise level with sire EBVS for SCS for the first ($p = 0.012$), second ($p < 0.001$), and third ($p < 0.001$) lactations.

Discussion

This study reports the link between DNA polymorphisms of *SPP1*, the number of milk immune cells and, potentially, the susceptibility to mastitis. These SNPs were identified by *in silico* search to be located in transcription factor recognition sites which factors are presumably involved in the Th1 immune response and in the Th2 regulation pathway. Indeed, one SNP abolished the SP1 recognition site, whereas another SNP affected the transcription binding factor IKAROS. All together, these findings support the genetic potential of these variants in terms of selection for the improvement of mastitis resistance in dairy cows.

BACKGROUND

Mastitis is an inflammatory condition of the mammary gland caused primarily by microorganisms, usually bacteria, that invade the udder, multiply and secrete toxic products that are very harmful to the host. In Canada, environmental mastitis (clinical mastitis) is most commonly caused by *Escherichia coli*. This infection is generally short taking a few days to be eliminated by the immune system, but the animal presents severe clinical signs that include inflammation of the udder, milk clots and altered behaviour (fever, loss of appetite). With annual costs for the herd of approximately \$180 per cow (BAR *et al.*, 2008), mastitis is still the most commonly occurring disease in Canadian dairy herds. These important losses to producers result not only from early culling and treatment costs, but also from the adverse effects of the decrease in production, and the need to discard milk that is unfit for human consumption because it is infected or contains antibiotic residues (BRADLEY, 2002; HUIJPS *et al.*, 2008).

The mammary gland is typically a sterile environment and, therefore, the entry of any foreign body usually triggers a localized immune response. The first line of defence against disease-causing microorganisms is the innate immune system, which induces mechanisms that are not pathogen species-specific (SORDILLO *et al.*, 1997). Innate immune cells in the mammary gland are comprised of macrophages, granulocytes, natural killer cells, and dendritic and mammary epithelial cells (RAINARD et RIOLLET, 2006). These cells have receptors that recognize motifs or pathogen-associated molecular patterns (PAMP) on the surface of microorganisms. For example, the lipopolysaccharides on the surface of Gram-negative bacteria such as *E. coli* become attached to the

phagocytic cells via Toll-like receptor 4 (TLR-4), whereas Toll-like receptor 2 (TLR-2) binds to Gram-positive motifs such as peptidoglycan or lipoteichoic acid on the surface of *S. aureus* (HIRSCHFELD *et al.*, 2000).

Recognition of an invading pathogen activates cellular reactions, leading to the secretion of inflammatory mediators called cytokines. These signalling molecules trigger cellular communication, chemotaxis and lymphocyte differentiation. The cytokines include inflammatory interleukins-(IL)-1 β , -6 and -12, tumour necrosis factor-alpha (TNF- α) and interferon-gamma (IFN- γ) (AKIRA et TAKEDA, 2004). Once phagocytic cells recognize and internalize pathogens, the cells present the pathogen's antigenic determinants to the T lymphocytes. Then these cells, in the presence of IL-12, differentiate into Th1 effector cells which are responsible for cell-mediated immunity. These Th1 cells produce inflammatory mediators such as IFN- γ , that enhance macrophage effectors functions against intracellular pathogens (NAGAI *et al.*, 2001).

Macrophages are the predominant cells in the healthy mammary gland (CONCHA, 1986). During intramammary infection, however, a release of inflammatory mediators, especially by macrophages, leads to the recruitment of neutrophils into infected quarters from the circulation. At this stage, these cells account for more than 90% of milk cells (LAHOUASSA *et al.*, 2007). The neutrophils are responsible for the eventual elimination of the pathogens. For example, activated neutrophils degranulate and produce/secrete bactericidal components, namely reactive oxygen species (ROS) (PEVERI *et al.*, 1988). The recruitment of neutrophils into the mammary gland causes an increase in somatic

cell count (SCC) that can reach more than 1,000,000 cells/mL during the course of an infection, whereas the SCC is normally less than 100,000 cells/mL in a healthy mammary gland (SORDILLO *et al.*, 1997).

Prevention and control of mastitis by improving the natural defence mechanisms is important not only for dairy producers but also for consumers, because of increased concerns about food safety, antibiotic use and animal welfare (RUPP *et al.*, 2007). One approach would be to define breeding objectives with increased weight of health-related traits in genetic selection (SORDILLO et STREICHER, 2002). Genetic selection to increase antibody responsiveness seems to be possible, but the acquired immune response traits have proven to be inconsistent indicators of udder health (BEGLEY *et al.*, 2009). Components of the adaptive immune system have been studied intensively, but there is still need for the development of efficient vaccine against pathogens that cause intramammary infection for bovine (RUPP *et al.*, 2007; WILSON *et al.*, 2007; WILSON *et al.*, 2007; WILSON *et al.*, 2009). Unlike the adaptive immune system, some mechanisms of the innate immunity are conserved throughout the animal kingdom and can thus be thought of as general mechanisms responsible for broad environmental responses. These evolutionarily conserved systems have been analyzed in detail and include, among others, the complement gene family (NONAKA et KIMURA, 2006) and the Toll-like receptors (TLR) (JAULT *et al.*, 2004). The parameters of innate immune responses can be used to study resistance to mastitis. These results can lead to the selection of breeding animals that carry favorable polymorphisms or alleles able to improve the resistance to infection of their offspring. (KADARMIDEEN *et al.*, 2006).

Genetic parameters such as heritability and phenotypic and genetic correlations are useful statistical tools for measuring the genetic component of a trait or group of traits. These genetic parameters do not require any knowledge about the number of genes involved as they are estimated from correlations of phenotypic data among relatives. The present study is based essentially on the somatic cell score (SCS), because data for clinical mastitis prevalence are still not available for the Canadian dairy population (OLDE RIEKERINK *et al.*, 2008). However, the usefulness of SCS as an indirect selection tool for reducing mastitis has been reported in several studies (HERINGSTAD *et al.*, 2000; MRODE et SWANSON, 2003; RUPP *et al.*, 2009; RUPP et BOICHARD, 2003). Most estimates of the genetic correlation between SCS and clinical mastitis range from 0.50 to 0.80 (LUTTINEN et JUGA, 1997; NIELSEN *et al.*, 1997; POSO et MANTYSAARI, 1996; RUPP et BOICHARD, 1999). In a recent study, the genetic correlation was observed to ranges from 0.55 to 0.93 (DE HAAS *et al.*, 2008). These are reasonably high values which suggest that SCC and mastitis occurrence are partly caused by the expression of the same trait. The SCC distribution is often highly skewed and it is usually transformed on a logarithm scale, as follows: $SCS = \log_2 (SCC/100,000) + 3$ (RUPP et BOICHARD, 2003). When used for genetic evaluation, bulls receive an estimated breeding value (EBV) based on the SCS records of their daughters. If dairy producers select bulls with a low EBV for SCS, this is expected to improve mastitis resistance in their herd (HERINGSTAD *et al.*, 2008; RUPP et BOICHARD, 1999; RUPP et BOICHARD, 2003). The SCS has now become the most important indicator associated with the health of the mammary gland, but little is known about the key factors which

regulate the number of somatic cells in milk from healthy cows. Thus, in the present study we investigated the early activated transcripts of immune cells of the lactating mammary gland in order to identify a key gene in the innate immune response to mastitis.

Osteopontin (or secreted phosphoprotein 1, *SPPI*) is a cytokine produced by macrophages and activated T cells (PATARCA *et al.*, 1989). Osteopontin has been described as an early component of the T cell activation mechanism. Enhanced in T cells, it recruits macrophages at the infection site and improves cell-mediated immunity (Th1) by inducing secretion of Th1 cytokines (CHABAS, 2005; CHABAS *et al.*, 2001; DENHARDT *et al.*, 2001; KONNO *et al.*, 2006). Osteopontin is a multi-faced protein (CHABAS, 2005; WANG et DENHARDT, 2008). It promotes bone remodelling (CAMPBELL *et al.*, 2003), wound healing and survival of stressed cells (GIACHELLI *et al.*, 1995), but is also associated with metastasis status and poor survival prognosis (PLUMER *et al.*, 2008; SINGHAL *et al.*, 1997). It was found associated with certain pathologies such as restenosis, formation of kidney stones, and autoimmune disease (WANG et DENHARDT, 2008), but also found to confer resistance to several intracellular pathogen infections through recruitment and activation of macrophages (WEBER *et al.*, 2002). In this study, we reported the detection of this abundant transcript, which is expressed early on during mastitis. Following the detection of this key gene in the mammary somatic cells isolated early during the immune response, we evaluated the association of *SPPI* with SCC, which is the most widely used indicator of mastitis (HERINGSTAD *et al.*, 2008). Identifying favourable *SPPI* alleles for mastitis resistance

would make it possible for dairy breeders to increase them in the Canadian Holstein population to potentially increase the natural resistance to mammary gland infection.

RESULTS

Experimental mastitis and detection of the candidate gene

An infectious dose of 94 ± 8 CFU of a fresh exponential culture of *E. coli* was used and produced a bacterial infection in all challenged quarters (Figure 1A) of the four lactating cows: groups 6 h ($n = 2$) and 12 h ($n = 2$). The front control quarters, which had been infused with saline, remained free of bacteria. There were no apparent signs of clinical infection at 6 h whereas infection was confirmed by 12 h. These two cows showed signs of clinical infection: clotting in milk, fever (40°C), and local redness along with pain after 12 h. The tumour necrosis factor-alpha (TNF- α) was detectable in milk at 9 h post-infection (Figure 1A). Increases in both SCC and granulocytes in milk were highly correlated between 6 and 9 h post-challenge (Figure 1B), which confirmed that granulocytes were the major invading cells in the mammary gland upon infection. During the same 6-to-9-h period, macrophages drastically decreased in proportion ($p < 0.05$; Figure 1B). Only 2 to 4% remained detectable whereas in the bacteria-free quarters, a significant level (51 to 56%) of macrophages was maintained. The inflection point for this longitudinal survey was 6 h. Indeed, no changes in the distribution of lymphocytes, macrophages and granulocytes in milk, between infected quarters (continuous lines) and uninfected quarters (dashed lines) were apparent in the first 6 h post-infection (Figure 1B). We thus monitored the molecular events prior to the increase of granulocytes (inflection point) in milk. The differential transcripts of milk immune cells were analysed at 5 h post-infection. One hundred clones from the cDNA library of milk immune cells were sequenced (Material and Methods). The *SPP1* transcript was identified using the Basic Local Alignment Search Tool (BLAST) to search against the National Center for

Biotechnology Information (NCBI) database. This induction of *SPP1* transcript was also observed in PBMC (peripheral blood mononuclear cells) challenged *in vitro* with heat-inactivated bacteria as described in Material and Methods. The increased *SPP1* transcripts abundance in PBMC was detected within 6 h (up to 4 fold by 3 h) by real-time RT-PCR in three independent assays (data not shown).

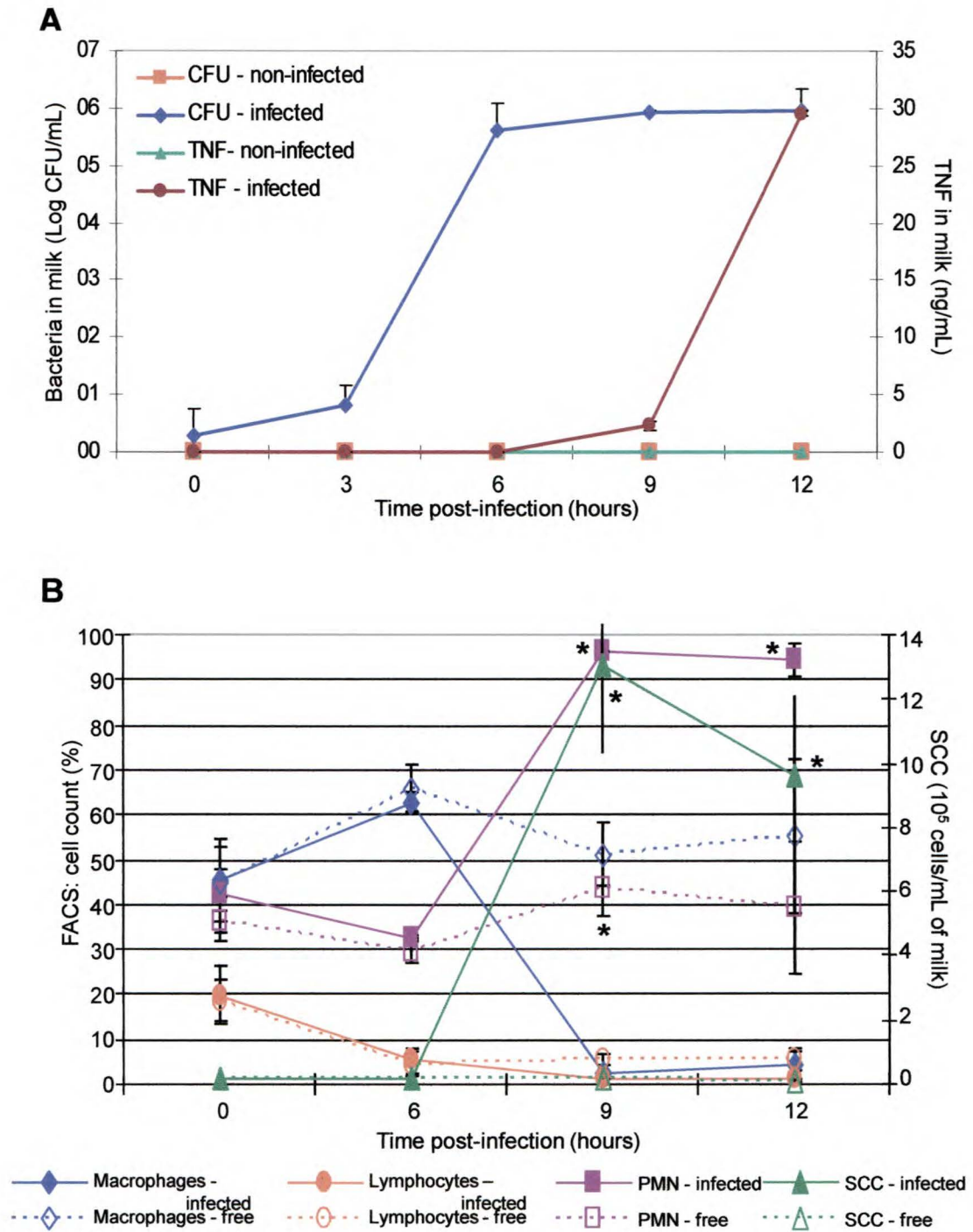


Figure 1. Experimental mastitis. Milk samples were analysed at different times for intramammary bacterial infection (panel A; CFU, left y-axis), inflammatory mediator

(panel A; TNF- α ; right y-axis), and for somatic cell profile (panel B). Data are expressed as mean \pm standard error of the mean. During the first 6 hours post-challenge, mean values of 4 cows are reported. By 6 hours, two cows were not sampled because they received antibiotics (Materials and Methods). Error bars, standard error of the means.

Analysis of the *SPP1* genomic sequence

Search for DNA polymorphisms was performed by selecting bulls with extreme EBV for SCS (Material and Methods). Each different genomic region of *SPP1* (promoter, 5' untranslated sequence [UTR], intron 1, exons 1–7 and 3' UTR) were sequenced as described in Materials and Methods. Comparison of the results of the electrophoregrams from the two groups presenting extreme EBV for the SCS trait uncovered differences in the allelic distribution of four nucleotides. Two single-nucleotide polymorphisms (SNPs) were found in the promoter. A novel SNP was identified and has been submitted to NCBI dbSNP on April 23rd 2009 (SNP submission number ss130452346). This novel transition SNP (*SPP1c*.-1301G>A) and the transition SNP *SPP1c*.-1251C>T were found respectively 1,301 and 1,251 nucleotides (nt) before the ATG (start) codon, which initiates translation from exon 2. The third transition SNP (*SPP1c*.-430G>A) was located in the first intron, 430 nt upstream from the start codon. Lastly, the transversion SNP (*SPP1c*.*40A>C) was found 40 nt downstream from the stop codon in exon 7 within the 3' UTR. No SNPs were found in the coding region. We scrutinized the electrophoregrams in the 2,428-to-2,419-nucleotide region upstream from the ATG gene corresponding to the insertion and deletion (INDEL) variation rs43702359 (NCBI dbSNP accession

number) which had been reported elsewhere (SCHNABEL *et al.*, 2005). No such variation was observed in the population sequenced.

SNPs and allelic frequencies

Genotypic and allelic frequencies of the four SNPs for the 578 bulls are summarized in Table 1. All SNPs were checked for conformance with the Hardy-Weinberg equilibrium, as determined by the Chi-square test. The frequency of the alleles varied in the population. Some alleles were present in low frequency, such as allele A (10.5%) for the SNP *SPP1c.-1301G>A*, which also explained the low abundance of homozygous bulls (1.0%) in the population tested. Homozygous bulls harbouring the genotype T for *SPP1c.-1251C>T* and genotype A for *SPP1c.-430G>A* were not frequent (6.4%), and the abundance of these alleles was 25.2% for both among the 578 genotyped bulls. These two SNPs were linked in the population tested: allele C for *SPP1c.-1251C>T* was linked to allele G for *SPP1c.-430G>A*, and *vice versa* (allele T linked to allele A). For the SNP *SPP1c.*40A>C*, 9.1% of the bulls were homozygous for allele C, a proportion of 30% in the population genotyped (Table 1).

Table 1. Genotype and allele frequencies of the SNPs detected in the bovine *SPP1* gene.

DNA polymorphisms	Genotype	Code	Frequency (%)		Allele	Frequency (%)	
<i>SPP1c.-1301G>A</i>	GG	-1	463	(80.1)	G	1035	(89.5)
	GA	0	109	(18.9)			
	AA	1	6	(1.0)	A	121	(10.5)
<i>SPP1c.-1251C>T</i>	CC	-1	324	(56.1)	C	865	(74.8)
	CT	0	217	(37.5)			
	TT	1	37	(6.4)	T	291	(25.2)
<i>SPP1c.-430G>A</i>	GG	-1	324	(56.1)	G	865	(74.8)
	GA	0	217	(37.5)			
	AA	1	37	(6.4)	A	291	(25.2)
<i>SPP1c.*40A>C</i>	AA	-1	287	(49.6)	A	813	(70.3)
	AC	0	239	(41.4)			
	CC	1	52	(9.1)	C	343	(29.7)

SNP associations

The EBVs for SCS at the first, second and third lactations and for over all lactations are reported according to the genotypes for *SPP1c.-1301G>A*, *SPP1c.-1251C>T*, *SPP1c.-430G>A* and *SPP1c.*40A>C* (Table 2). Allele G of *SPP1c.-1301G>A* had a favourable impact on SCS EBV at any lactation ($p < 0.001$). The SNP *SPP1c.-1251C>T* and *SPP1c.-430G>A* did not have an effect on SCS EBV (global comparison – over all lactations), but genotypic comparison showed a significant difference for the third lactation only between homozygous and heterozygous bulls for the dominant allele ($p = 0.046$; data not shown). The statistical analysis also revealed a stronger association between *SPP1c.*40A>C* and EBVs for SCS as the number of lactations increased: first lactation (SCS1; $p = 0.183$), second lactation (SCS2; $p = 0.014$) and third lactation (SCS3; $p = 0.004$).

The estimated average allele substitution effects are presented in Table 3. No significant association of SNP *SPP1c.-1301G>A* was detected with SCS in any of the lactations. The SNP *SPP1c.-1251C>T* and *SPP1c.-430G>A* were significant at a comparison-wise level in association with EBVs for SCS ($p = 0.014$), SCS1 ($p = 0.035$), and SCS2 ($p = 0.023$). The estimated substitution effect was about the same for SCS3 but the larger SE crushed the statistical validity ($p = 0.056$). The allele substitution effects for *SPP1c.-1251C>T* for over all, first, and second lactations are respectively 0.117 ± 0.047 , 0.097 ± 0.046 and 0.118 ± 0.052 . Thus, the increase in SCS for allele T over allele C corresponded to 33% of the SD for SCS EBV. The same effects on EBV for SCS were found for *SPP1c.-430G>A*, but the negative allelic substitution effect carried by allele A had the

opposite effect (reduced the EBV for SCS). Although it was not significant for the first lactation (SCS1; $p = 0.121$), the 3' UTR SNP *SPP1c*. *40A>C was associated with EBVs for SCS2 ($p = 0.038$), SCS3 ($p = 0.045$) and over all lactations ($p = 0.023$); the corresponding allele substitution effects for this SNP were -0.103 ± 0.049 , -0.112 ± 0.056 and -0.102 ± 0.045 , respectively, meaning that allele C reduces SCS over allele A.

Table 2. Effects of polymorphisms in the bovine *SPP1* gene on EBVs for SCS for the first, second, third, and over all lactations.

SNP	Least squares mean ^a			(± SEM) ^a	p value
<i>SPP1c.-1301G>A</i>	GG	GA	AA		
SCS	2.98 ^b	3.16 ^c	3.38 ^c	(±0.14)	<.001
SCS1	2.98 ^b	3.10 ^c	3.25 ^{b,c}	(±0.13)	<.001
SCS2	2.96 ^b	3.14 ^c	3.39 ^c	(±0.15)	<.001
SCS3	2.99 ^b	3.16 ^c	3.50 ^c	(±0.16)	<.001
<i>SPP1c.-1251C>T</i>	CC	CT	TT		
SCS	3.04	2.97	3.02	(±0.06)	0.067
SCS1	3.02	2.98	3.02	(±0.05)	0.446
SCS2	3.03	2.96	3.00	(±0.06)	0.155
SCS3	3.06 ^b	2.97 ^c	3.01 ^{b,c}	(±0.07)	0.057
<i>SPP1c.-430G>A</i>	GG	GA	AA		
SCS	3.04	2.97	3.02	(±0.06)	0.067
SCS1	3.02	2.98	3.02	(±0.05)	0.446
SCS2	3.03	2.96	3.00	(±0.06)	0.155
SCS3	3.06 ^b	2.97 ^c	3.01 ^{b,c}	(±0.07)	0.057
<i>SPP1c.*40A>C</i>	AA	AC	CC		
SCS	3.06 ^b	2.97 ^c	2.98 ^{b,c}	(±0.05)	0.004
SCS1	3.03	2.98	2.98	(±0.05)	0.183
SCS2	3.05 ^b	2.95 ^c	2.97 ^{b,c}	(±0.05)	0.014
SCS3	3.09 ^b	2.97 ^c	2.97 ^{b,c}	(±0.06)	0.004

^aThe least square means are the adjusted means of the EBVs for the SCS for bulls which are grouped by genotype for each SNP and calculated for the respective lactation (over all, first, second or third lactations). SEM standard error of the mean.

^{b,c} Means (within a line) without a common superscript letter differ from each other at the 5% level of significance.

Table 3. Association of the SNPs in the *SPP1* gene with EBVs for SCS^a.

Polymorphisms	Average allelic substitution effect											
	SCS			SCS1			SCS2			SCS3		
	Average allele effect ± SE (<i>p</i> value)											
<i>SPP1c.-1301G>A</i>	0.047	± 0.030	(0.121)	0.033	± 0.030	(0.280)	0.047	± 0.034	(0.165)	0.035	± 0.038	(0.362)
<i>SPP1c.-1251C>T</i>	0.117	± 0.047	(0.014)	0.097	± 0.046	(0.035)	0.118	± 0.052	(0.023)	0.112	± 0.059	(0.056)
<i>SPP1c.-430G>A</i>	- 0.117	± 0.047	(0.014)	- 0.097	± 0.046	(0.035)	- 0.118	± 0.052	(0.023)	- 0.112	± 0.059	(0.056)
<i>SPP1c.*40A>C</i>	- 0.102	± 0.045	(0.023)	- 0.068	± 0.044	(0.121)	- 0.103	± 0.049	(0.038)	- 0.112	± 0.056	(0.045)

^a EBVs are calculated for the respective lactation: over all (SCS), first (SCS1), second (SCS2), and third (SCS3) lactations.

Effect of *SPP1* SNPs for production traits

The average substitution effect of the SNP *SPP1c.-1301G>A* was significant for milk yield from the second ($p = 0.027$) and third ($p = 0.046$) lactations. Substitution of allele G over allele A decreased the EBV for milk yield by the equivalent of 178 kg and 165 kg, respectively (Table 4). Although no significant effect was observed for fat or protein yield (kg), the same allele G had a favourable effect on the EBVs for the fat and protein percentages. Protein percentages were associated with the SNP in the second ($p = 0.030$) and third ($p = 0.036$) lactations ($0.03\% \pm 0.01$), whereas the effects for fat percentages were observed for all three lactations, as follows: $0.08\% \pm 0.03$ ($p = 0.004$), $0.09\% \pm 0.03$ ($p = 0.012$) and $0.08\% \pm 0.03$ ($p = 0.009$), respectively. None of the three other SNPs was found to have a significant allelic substitution effect when associated with the EBVs for production traits (data not shown) or any significant effect using the regression model (Additional file 1). However, all three SNPs were associated with EBVs for fat yield and fat percentage ($p < 0.05$) (Additional table 1).

Table 4. Association of the SNP *SPP1c.-1301G>A* in the *SPP1* gene with EBVs for production traits.

Trait	Average allelic substitution effect											
	EBV unit \pm SE (<i>p</i> value)											
	Over all lactation			1st lactation			2 nd lactation			3 rd lactation		
Milk yield (Kg)	-116	\pm 78	(0.142)	-177	\pm 91	(0.052)	-178	\pm 80	(0.027)	-164	\pm 82	(0.046)
Fat yield (Kg)	2	\pm 3	(0.500)	3	\pm 3	(0.289)	1	\pm 3	(0.658)	2	\pm 3	(0.485)
Protein yield (Kg)	-2	\pm 2	(0.420)	-3	\pm 3	(0.175)	-3	\pm 2	(0.167)	-3	\pm 2	(0.251)
Fat %	0.05	\pm 0.03	(0.059)	0.09	\pm 0.03	(0.004)	0.08	\pm 0.03	(0.012)	0.08	\pm 0.03	(0.009)
Protein %	0.02	\pm 0.01	(0.090)	0.02	\pm 0.01	(0.060)	0.03	\pm 0.01	(0.030)	0.03	\pm 0.01	(0.036)

Haplotype analysis

Table 5 shows the estimated population haplotype frequencies, comparing two different algorithms provided by the HAPROB and Haploview analysis methods. Both methods reported that four haplotypes were more likely to be present in the population tested. However, HAPROB analysis was more sensitive for detecting low abundant alleles. Block H1 (GCGA) was the most frequent haplotype (59.2%), whereas block H5 was detected in only six offspring among the 578 bulls. Blocks H2, H3 and H4 had frequencies equal to 0.24, 0.10 and 0.05, respectively. The remaining haplotypes, H6 to H13, had frequencies less than 0.2% and were pooled together (into H6) for statistical analysis. Table 6 reports the genotypes of both alleles from all 578 bulls, displayed using the haplotype blocks present in the population (Table 5). The EBVs (least mean squares) for all traits are reported, according to their homozygosity or heterozygosity, sorted by increasing value of the EBV for SCS for the over all lactations. Analysis revealed statistically significant differences between haplotype groups at a comparison-wise level with EBVs for SCS1 ($p = 0.012$), SCS2 ($p < 0.001$), SCS3 ($p < 0.001$) and over all lactations ($p < 0.001$). For production trait, analysis revealed statistically significant differences between haplotype groups at a comparison-wise level with EBVs for fat ($p = 0.044$) and fat percentage ($p = 0.043$) (Table 6), and for protein percentage for the first and second lactations only (data not shown). None of the haplotypes produced a significant allele substitution effect on EBV for SCS or for the production traits (Table 7 or Additional table 1).

Table 5. Estimated haplotype block and population frequencies of the *SPP1* locus.

Haplotype	<i>SPP1c.-</i> <i>1301G>A</i>	<i>SPP1c.-</i> <i>1251C>T</i>	<i>SPP1c.-</i> <i>430G>A</i>	<i>SPP1c.*40A>C</i>	Frequency ^a	Frequency ^b
H1	G	C	G	A	0.59230	0.599
H2	G	T	A	C	0.23976	0.252
H3	A	C	G	A	0.10267	0.105
H4	G	C	G	C	0.05079	0.045
H5	G	T	A	A	0.00704	
H6	A	T	A	C	0.00164	
H7	G	T	G	C	0.00125	
H8	G	C	A	C	0.00123	
H9	A	C	G	C	0.00107	
H10	G	C	A	A	0.00106	
H11	G	T	G	A	0.00104	
H12	A	C	A	C	0.00003	
H13	A	T	G	A	0.00003	
H14	A	T	G	C	0.00003	
H15	A	C	A	A	0.00003	
H16	A	T	A	A	0.00003	

^aAnalysis were performed using HAPROB (BOETTCHER *et al.*, 2004)

^bAnalysis were performed using Haploview (BARRETT *et al.*, 2005), version 4

Table 6. Haplotype frequencies of the bovine *SPPI* gene and estimated effects on EBVs for SCS^a and for production traits.

Genotype		Frequency		Least squares mean								
Allele 1	Allele 2	(%)		SCS	SCS1	SCS2	SCS3	Milk yield (Kg)	Fat yield (Kg)	Protein yield (Kg)	Fat %	Protein %
H4 (GCGC)	H4 (GCGC)	2	(0.4)	2.81	2.9	2.74	2.79	-594	0	-17	0.22	0.02
H1 (GCGA)	H4 (GCGC)	30	(5.2)	2.87	2.90	2.85	2.88	-238	-6	-3	0.03	0.04
H2 (GTAC)	H4 (GCGC)	13	(2.2)	2.88	2.88	2.92	2.88	-333	-8	-8	0.04	0.03
H1 (GCGA)	H2 (GTAC)	170	(29.4)	2.94	2.96	2.92	2.94	-128	1	-0.7	0.07	0.04
H3 (ACGA)	H4 (GCGC)	5	(0.9)	3.01	3.04	3.03	2.96	296	25	8	0.15	-0.01
H1 (GCGA)	H1 (GCGA)	205	(35.6)	3.02	3.00	3.01	3.05	42	-2	1	-0.02	0.001
H2 (GTAC)	H2 (GTAC)	37	(6.4)	3.02	3.02	3.00	3.01	-99	-13	-2	-0.09	0.02
H4 (GCGC)	H5 (GTAA)	6	(1.0)	3.03	3.01	3.03	3.05	-244	4	-8	0.14	0.005
H1 (GCGA)	H3 (ACGA)	76	(13.2)	3.16	3.09	3.13	3.17	-139	2	0.1	0.07	0.05
H2 (GTAC)	H3 (ACGA)	28	(4.8)	3.17	3.15	3.18	3.2	-198	-7	-2	0.01	0.04
H3 (ACGA)	H3 (ACGA)	6	(1.0)	3.38	3.25	3.39	3.50	-517	-11	-10	0.08	0.07
± SEM				± 0.24	± 0.22	± 0.26	± 0.28	± 567	± 19	16	± 0.21	± 0.08
<i>p</i> value				<.001	0.012	<.001	<.001	0.236	0.044	0.745	0.043	0.078

^a EBVs are calculated for the respective lactation: over all (SCS), first (SCS1), second (SCS2), and third (SCS3) lactations

Table 7. Estimated haplotype effects on EBVs for SCS.

Haplotypes	Estimated haplotype effect											
	SCS			SCS1			SCS2			SCS3		
	EBV unit \pm SE (<i>p</i> value)											
H1 (GCGA)	0.118	\pm 0.268	(0.660)	0.034	\pm 0.261	(0.896)	0.120	\pm 0.295	(0.685)	0.173	\pm 0.333	(0.604)
H2 (GTAC)	0.132	\pm 0.274	(0.629)	0.061	\pm 0.266	(0.818)	0.136	\pm 0.300	(0.650)	0.174	\pm 0.340	(0.609)
H3 (ACGA)	0.169	\pm 0.277	(0.541)	0.071	\pm 0.270	(0.793)	0.177	\pm 0.305	(0.562)	0.209	\pm 0.345	(0.545)
H4 (GCGC)	0.023	\pm 0.276	(0.935)	-0.030	\pm 0.269	(0.913)	0.025	\pm 0.303	(0.933)	0.071	\pm 0.343	(0.836)
H5 (GTAA)	0.212	\pm 0.352	(0.547)	0.090	\pm 0.339	(0.790)	0.247	\pm 0.382	(0.518)	0.285	\pm 0.433	(0.512)

DISCUSSION

The objective of this work was to identify one candidate of the innate immune response, to define the association of the genetic variants with the immune cells in milk, but also to evaluate their impact on other traits related to dairy production. Osteopontin has been described as an early component of the T cell activation mechanism and was also detected in the immune cells in the present study during the first hours of *E. coli* infection. This confirms the results of other studies which show the importance of osteopontin in inflammatory responses. The expression of *SPP1* is enhanced in T cells during bacterial infection in order to recruit macrophages to the infection site and to improve cell-mediated immunity (Th1) by increasing the secretion of Th1 cytokines (CHABAS, 2005; DENHARDT *et al.*, 2001; KONNO *et al.*, 2006). Indeed, knockout *SPP1*^{-/-} mice have shown significantly impaired Th1 immunity to viral and bacterial infections with diminished production of interleukin-12 (IL-12) and interferon-gamma (IFN- γ) and elevated production of interleukin-10 (IL-10) (ASHKAR *et al.*, 2000). However, no studies have been carried out to correlate osteopontin with mastitis.

Innate immune responses are activated by a cross-species conserved signalling pathway. Study of this activation could lead to the identification of candidate genes for disease resistance. The detection of favourable allelic variants of the innate immune response genes will make it possible to select progeny with a more efficient immune system. Because of the potential immunological role of *SPP1* in the milk of cows diagnosed with mastitis, the next step was the validation of *SPP1* genetic variants that would explain the SCS observed in the dairy population. Indeed, although SCS in milk is an indirect

measure of cow health status, SCS is considered to be the most widely used biological marker of clinical mastitis in lactating cows (DE HAAS *et al.*, 2008). We thus found polymorphisms in the genomic sequence of *SPP1* that influence the number of somatic cells in milk and, potentially, influence their susceptibility to mastitis, although this latter assumption remains to be validated. Through the use of animals with extreme EBV for SCS, a novel SNP was discovered in the promoter region of *SPP1* (*SPP1c.-1301G>A*), 50 nucleotides (nt) upstream from the SNP *SPP1c.-1251C>T*.

The 5' UTR SNP *SPP1c.-1251C>T* and *SPP1c.-430G>A*, and the 3' UTR SNP *SPP1c.*40A>C* were also identified both in the present study and in a previous one (SCHNABEL *et al.*, 2005). These DNA polymorphisms were not investigated for an association with SCS. However, these authors found one DNA polymorphism (T₉/T₁₀ INDEL) that had an effect on the fat and protein percentage traits, based on the results of 167 bull sires, but was not associated with SCS (SCHNABEL *et al.*, 2005). This INDEL polymorphism, which corresponds to polyT tract alleles of either nine or 10 thymines (T₉/T₁₀ INDEL), is absent from the 100 bulls sequenced in the present study, and also from the mouse (HIGASHIBATA *et al.*, 2004), human and swine *SPP1* promoter (see sequence alignment in Additional file 2A). The bovine *SPP1* region containing this polymorphism (also -1,301 and -1,251 nt) was aligned with other mammals using the CLUSTALW algorithm (Additional file 2A). This region does not contain any transcription factor (TF) motifs (data not shown). The T₉/T₁₀ INDEL polymorphism may not associate with TF. It is located 1,119 nt upstream from the novel *SPP1c.-1301G>A*, is absent from sequenced individuals selected, and thus may not be a functional SNP for the

SCS trait. Therefore, we cannot exclude this T₉/T₁₀ INDEL polymorphism from the Canadian population, since the present experimental design does not address differences among bulls with extreme EBVs based on production traits (e.g. fat).

We searched for cross-species similarity in regions of the genome containing the SNP detected in the present study. Both of the SNPs in the promoter are very similar to other species (Additional figure 1: B and C). Interestingly, these regions included a “GC-rich” area identical to both the mouse and the human regions. Among the potential functional factors that could explain the impact on the EBV for the SCS, IKZF2 (IKAROS family zinc finger 2) and SP1 are two TFs that recognize binding sites at or in the vicinity of the SNP (Figure 2). The TF SP1 binds specifically to GC box DNA within the 5' flanking promoter sequences for promoting eukaryotic transcription (JONES *et al.*, 1987; YOKONO *et al.*, 1998). Specifically, the TF SP1 binds to the *SPP1* promoter and is involved in Th1 immune responses (HUMMELSHOJ *et al.*, 2006). The TF IKZF2 is a regulator of Th2 responses (QUIRION *et al.*, 2009). In that way, allele G of *SPP1c.-1301G>A* abolishes IKZF2 recognition (Figure 2). Thus, impaired IKZF2 binding would have the consequence of promoting a Th1 immune response in the absence of a Th2 polarizing effect on naive immune cells (QUIRION *et al.*, 2009). Impaired IKZF2 binding could also facilitate the access of SP1 (Th1 response) through its binding activity, which is present in the vicinity of *SPP1c.-1301G>A* (Figure 2). This hypothesis is supported by the fact that allele A is associated with an elevated SCS (Table 3), SCS having been associated with increased mastitis incidence (DE HAAS *et al.*, 2008).

Indeed, an inefficient Th1 response or abrogated innate immunity predisposes cows to environmental mastitis.

TTCATGGGCCCTCTAGATGCCCTTCCAGGATGCTGGAAGTCAGTGCTATGAACAAAAAAGATAGTTAGTGATATTGTACATA
 AGTAATGTTTTAACTTTAACTAGCAGGGTAGTGGGTGTTTGTGTGCGTGTGCGCGTGTGTGTGTGCCTGTGTTTGTCTGTGA
 c-Myb
 CCACAAAACCAGAGGGGGAAGTGTGGGAACAAGTGGGCTGGGTAGTGGCAAAATGCCCATGACACATCTCTCCGCCCCCTGT
 IKZF2 SP1 SP1 STRE
 SPP1c.-1301G>A SPP1c.-1251C>T
 GTAGGTGGAGAGCATCTGGAGCAGCCTTTAAATTCTGGGAGATCCTGGTTGTGAGCAGCAGGGAGAGCAGGCCAGGAGGGCAG
 Lyf-1 AP-4
 CACTGAGCACTGCATCAGCATCACAGGGGACTGGACTCTTCTGCGCCGCCGAGACCAAGGTAAGCCTGCAGTTTGCTACAGAC
 TCCTGTCTCTCTGTGCGCTGCCTCAITTCATTGGGAAGGTCAATTTGTAAGSAAAAGAGTATAATGGTAACACTGTTAATC
 AP-1 IKZF1/2
 AGACTTGAGTTGTTCTTGTCTTTGAATATGCATGCCAGATCCAGGGCTGATGTCTCTGCAGGAAAGGTGGCCGGTTATTTTGA
 Oct-1
 AAGACAGTCGAATATAAACTTGAAAATATTTCCATGGAGTCCTCAAAAGAATTGAGACTACTTTTTTTCAGTCAGGAAATAAA
 AGAAAAATTCTATGCCCTTTTGGGATGATTGTATATACATCAITTTAATGAATAGATGACCATGGGATATTTTAAAGGAAAAAT
 IKZF2 TCF11 NF-AT
 GCTTTTTAGTATTCAATATAACCTGACGTTAAATGCTAATTTTTAGTGATTTGATTTATCCCTTTTTCAAGGAAAAAAAAAAAA
 ATF CRE-BP STATx
 CCGTTTCTGAATATTTTACCTCTGTATTTAGCTATTAAAATTTCACCCAAATATCTATATGATACTGTTTAGACTTACAAAT
 AGAAAAGCTGTTGACTTCAGTGTTTTCTTTTCAITTCAAACTTTTAGAATACCTTGACTTACTAACCTTAGAGACAGCTACA
 TTACACCTAACTAATACCTTTTAAATAATTTAAAATCACATTTGAAATGCATGTTGGAAAATGGAGACAACAAGTTTCTCTTT
 NF-AT ISRE
 SPP1c.-430G>A
 CTTATTTTTATCTTCTCTCTTCATGTTTTTCTTCTCTGAAAAGTAAATATTCTCATTCTTGCTTTATTATTTTAAATCAATTA
 CTGCTGATCTGTTTTTAGGTTTAGATAGCTGGAGATATCAGGTAGTAATGGTGTAAATCTCTGAAACTCTAAATGTTAAAGTCG
 AATAAATATAGATTTGTAAAATTCCTCTCTCCCTTGCCTAATAGTGAGAGATGGAAAATAGAGGTGGCAGTACAAATATTAAC
 TCAAAAGATCATAATATTTAAAAGAAATTAGTGGAGTGTTTCCACACAAAATACATATTTATTTGTGATGATTTTGTAAATGTG
 GATA-3 CCAAT
 GTAGCCTAAAAAAGTATCACTGTTTTGACCTTAGAAAAGATAAAATATTTCTTACAAAATATTTTGCAGGAAAAATCATTAC
 GATA-1/2/3 STATx NF-AT
 CATGAGAATTGCAGTGATTT GGTCAATTGAAAGGAGAAAATACAATTTCTTACTTTGCTTTTGTAGTAAAAAGAAAAGGA
 +1 *1 SPP1c.*40A>C

Figure 2. Nucleotide sequence of the 5' upstream region (GenBank accession No. AY878328) of the bovine osteopontin gene *SPP1*. The 5' region is indicated in uppercase letters, and position +1 (translation initiation codon) is indicated in italic uppercase letters until position *1 (translation termination codon). SNPs are in bold. Putative binding sites (found with <http://motif.genome.jp>) are shown in grey or underlined when co-localized: octamer factor 1 = Oct-1; TCF11/KCR-F1/Nrf1 homodimers = TCF11; LyF-1 = Lyf-1; c-Myb = c-Myb; stimulating protein 1 = SP1; stress-response element = STRE; AP-1 binding site = AP-1; activator protein 4 = AP-4; IKAROS family zinc finger 1 (Ikaros) = IKZF1; IKAROS family zinc finger 2 (Helios) = IKZF2; nuclear factor of activated T-cells = NF-AT; CRE-binding protein 1/c-Jun heterodimer = CRE-BP; signal transducers and activators of transcription = STATx; C/EBPalpha CCAAT/enhancer binding protein alpha = CCAAT; GATA-binding factor 1 = GATA-1; GATA-binding factor 2 = GATA-2; GATA-binding factor 3 = GATA-3; interferon-stimulated response element = ISRE; activating transcription factor = ATF.

An additional SP1 recognition site was found in the location of *SPP1c.-1251C>T*, with allele T abolishing the SP1 GC box recognition (Figure 2). This TF binding site has been found to colocalize with the human “-66T” SNP, which is referred to as the human *SPP1* transcription starting site (HUMMELSHOJ *et al.*, 2006). The authors, Hummelshoj et coll., confirmed by electrophoretic mobility shift assay that recognition of the TF SP1 is influenced by the presence of DNA polymorphism. Furthermore, using a luciferase assay reporting the *SPP1* promoter, they observed increased activity with allele T compared to allele C for the corresponding -66 T/G SNP (HUMMELSHOJ *et al.*, 2006). Alignment of human and bovine sequences revealed that the -66 T/G SNP is located 7 nt from the bovine SNP *SPP1c.-1251C>T*, both within the TF SP1 binding site. Although it remains to be confirmed, the influence of the SNP *SPP1c.-1251C>T* on the regulation of the bovine promoter is highly plausible. Indeed, the regulation of promoters based around the Sp family binding protein has been observed in a number of housekeeping genes. These factors act as transcriptional activators in mammalian cells (BLACK *et al.*, 2001). Since SP1 recruits the basal transcription machinery and controls the rate of transcription (GILL *et al.*, 1994; SUSKE, 1999), it thus explains the constitutive secretion of osteopontin in several biological fluids (blood, semen, serum, bile) and its distribution in a very broad variety of tissues (SODEK *et al.*, 2000). Our results suggest that the absence of the SP1 DNA binding site would impair or reduce the transcription activity of the *SPP1* promoter. Unless validation of the *SPP1* transcript or osteopontin protein from cows harbouring allele C or T *SPP1c.-1251C>T* is performed (haplotypes H2 and H5 contained an abolished SP1 binding site [Table 6]) the veracity of the tangible impact of *SPP1c.-1251C>T* on bacterial infection remains unknown. Further study by *in vitro* assay

using reporter genes and binding shift assays could help elucidate the impact of promoter variants on transcription activity.

Lastly, two other binding sites, the nuclear factor of activated T cells (NF-AT) and the interferon-stimulated response element (ISRE), were positioned close to *SPP1c*.-430G>A (-7 and +2 nt, respectively), located in the first intron. The NF-AT promotes cell proliferation and the expression of inflammatory cytokines, such as *IL-6* and *SPP1* (ZETTERQVIST *et al.*, 2008), whereas ISRE would regulate the expression of *SPP1* (LI *et al.*, 2003). A polymorphism in this region could potentially affect the binding of these two TFs and influence the rapidity of the response of *SPP1* to infection. Therefore, functional studies are required to interrogate the significance of this SNP with TF binding and gene expression.

In an association study, neither *SPP1c*.-1251C>T nor *SPP1c*.-430G>A was found to be associated with SCS (Table 2). We found that these two are linked (i.e. C/G or T/A on the same allele; data not shown), which could explain why only heterozygous animals (CT genotype for *SPP1c*.-1251C>T and GA genotype for *SPP1c*.-430G>A), when compared to their respective homozygotes, had an impact on SCS. This was also shown by their opposite allelic substitution effects (Table 3). Therefore, both allele C for *SPP1c*.-1251C>T and allele A for *SPP1c* 430G>A, which are linked to G and T, respectively, would be required to compensate for the negative effect of the second allele.

As innate immunity is not an adaptive immune response, an association study regarding the genetics should not be modulated as time passes. In other words, immune response should be maintained, irrespective of the number of times the pathogen is encountered. Indeed, this constant trend (for statistically significant values) was observed regarding the allele substitution effect (Table 3), which means that cows harbouring the favourable allele do not benefit (i.e. there is no decrease of the SCS value) in later parities from these *SPP1c.-1251C>T* and *SPP1c.-430G>A* SNPs. The SNP *SPP1c.-1301G>A* was only found to be associated with SCS in the regression model (Table 2). However, it may also have an impact that was not revealed by the allele substitution effect. Because of the discrepancy in the abundance of both alleles (89.5% versus 10.5%; Table 3), the substitution effect may not be statistically significant.

For the fourth *SPP1c.*40A>C* SNP, it is less clear how the 3' UTR DNA polymorphisms affect *SPP1*, although a more general mechanism such as microRNA would affect transcript stability. We cannot exclude that *SPP1* would be relatively more stable than other transcripts in milk cells. Although amplitude of the *SPP1* induction was smaller than *TNF* transcript, both were correlated as the bacterial challenge of the PBMC progressed (data not shown). The TNF- α is an important inflammatory mediator involved in neutrophil recruitment (OVIEDO-BOYSO *et al.*, 2007; RIOLLET *et al.*, 2000) and *SPP1* might activate or maintained the Th1-Th2 balance. Thus *SPP1* could be either an upregulated gene or stabilized transcript. MicroRNA are an important class of regulatory RNA that repress animal genes by preferentially interacting with complementary sequence motifs in the 3' UTR of target mRNA (MAJOROS et OHLER, 2007). The

importance of the region is further highlighted by the presence of the *SPP1c.*40A>C* SNP within two other bovine species. The presence of this DNA polymorphisms was detected within the Guernsey (n = 42) and Jerseys (n = 81) cows (data not shown). Whereas both human and mouse 3' UTR of *SPP1* are the target of several microRNA, retrieved by an *in silico* search (<http://www.microrna.org/>; data not shown), this hypothesis remains to be verified for the bovine *SPP1* 3' UTR sequences.

In previous studies, nine DNA polymorphisms were found within eight sires sequenced for the *SPP1* gene. Whereas one SNP was associated with production traits (fat and protein yield and percentage, milk yield), this “*OPN3909*” was not found associated with SCS (SCHNABEL *et al.*, 2005). The main reason may be inherent to the selection of the Holstein dairy cattle population, which in our study was based on extreme EBV for SCS from a directory of 6,453 bulls (Canadian Dairy Network database). How *SPP1* may affect fat yield and fat percentage in bovine milk remains an open question. In humans, a significant positive correlation was found between osteopontin levels and body fat as well as circulating concentrations of total cholesterol (GOMEZ-AMBROSI *et al.*, 2007). Osteopontin is also associated with cholesterol gallstone formation in human and mouse (ICHIKAWA *et al.*, 2009). The specific role of osteopontin beyond its immune role in milk warrants further investigation. From a genetic perspective, it is well known that health and disease may affect other performance traits such as longevity and fertility among others. Therefore, genetic factors affecting health trait may impact other traits. For bull fertility, the EBV for the male fertility index is reported as the non-return rate trait (Canadian Dairy Network database). Among the genotyped bulls, we did not find

significant statistical association of the four *SPP1* SNP with the EBV of the non-return rate (data not shown). Even though osteopontin was reported to influence bull fertility (CANCEL *et al.*, 1997; ERIKSON *et al.*, 2007), these *SPP1* variants were not found associated with male fertility (data not shown). We cannot exclude that a genetic association could be observed using bulls with extreme EBV for the male fertility index. Therefore, further investigations are still required before suggesting favourable allele for the innate immune system, such as how the genetic *SPP1* variants may influence male fertility or milk composition (e.g. concentration of cholesterol). In fact, milk is used for human consumption and an increase of the concentration of cholesterol may not be valuable.

In the present study, we found a strong correlation between *SPP1* and SCS, and we identified potential functional sites in the promoter of the gene. The different promoter haplotypes have been cloned, and the relevance of the functional sites will be studied in order to elucidate the molecular mechanisms responsible for the abundance of milk somatic cells in the Holstein dairy population as well as to depict the immune response to environmental stimuli.

CONCLUSIONS

This study reports the link between DNA polymorphisms within the innate immune *SPP1* gene, the number of milk immune cells and, potentially, susceptibility to mastitis. Indeed, one SNP abolished the SP1 recognition site, whereas another SNP affected the transcription binding factor IKAROS. These SNPs, located in the promoter, are potentially involved in the Th1 immune response and in the Th2 regulation pathway since they were identified by in silico search to abolish these transcription factor recognition sites. Because these SNPs are associated with the level of immune (somatic) cells measured in milk which is associated with clinical mastitis and because of their potential implication in the Th1–Th2, all together, these findings suggest the genetic potential of these variants in terms of selection for improving mastitis resistance in dairy cows. But osteopontin is not only involved in regulatory mechanisms of the immune response but also in mammary gland development and milk secretion. Thus, before recommending any *SPP1* haplotype for genetic selection, the beneficial role of these genetic forms would require further investigation.

METHODS

Animals

Twenty multiparous Holstein cows were selected from surrounding dairy farms based on several criteria. All cows were from different dam and sire families, and had low SCC ranging from 5×10^4 to 1×10^5 cells/mL, no past history of mastitis, tested negative for bovine virus diarrhoea, leucosis, neospora and mycoplasmosis, and were found to be free from bacteria in aseptically collected milk samples. Milk was aseptically sampled from each quarter every week starting at calving until the end of the trial period (110 to 170 days of lactation). Four cows were kept for the study based on these criteria: no calving associated problem and bacteriologically negative milk samples with SCC values below 1×10^5 cells/mL across lactation. The cows were transferred to the level 2 biosecurity facility one week before the challenge. They were handled according to the Guide for the Care and Use of Agricultural Animals by the Institutional Animal Care and Use Committee at Agriculture and Agri-Food Canada's Dairy and Swine Research and Development Centre (Document 188).

Preparation of bacteria and intramammary challenge

Escherichia coli strain SHY97-3923-2, previously isolated from a clinical case of bovine mastitis and kindly provided by the Laboratoire provincial de pathologie animale (St-Hyacinthe, Quebec, Canada), was used for the intramammary challenge. An antibiotic susceptibility test was validated on the strain (Biovet, St-Hyacinthe, Quebec, Canada). The bacteria were cultivated in tryptic soy broth (TSB) and the fresh starter was incubated for approximately 3 h to bring it to an exponential growth phase (0.6 to 0.7 OD). The

culture was then centrifuged, washed once in pyrogen-free saline (PFS) and re-suspended following an established serial dilution protocol to get a final concentration of 1,000 CFU/mL. The concentration was confirmed by plating 200 μ L of three independent dilutions on TSA. The 3-mL volume was injected in the challenged glands via the teat canal immediately after the morning milking in the left and right rear quarters of the mammary gland (n = 4 cows). As controls, the right and left front quarters of each cow were infused with 3 mL of sterile saline solution. All the quarters were then massaged to ensure dispersal of the inoculum. Bacteriological analysis was conducted by plating 200 μ L of each milk sample from each quarter onto TSA overnight at 37°C to determine the number of colony-forming units over the challenge.

Clinical signs, collected samples and assays

Antibiotic treatment (Borgal; Hoechst, Montreal, Canada) and pain relief medicine (Anafen; Merial, Baie d'Urfé, Canada) were administered following 6h (n = 2 cows) and 12 h (n = 2) according to the Guide for the Care and Use of Agricultural Animals. Rectal temperature and general state were monitored for each cow every 3 h. The SCC in milk samples were analyzed by the Dairy Production Centre of Expertise (Valacta, Montreal, Quebec, Canada) and provided values on crude milk. All contaminated samples were processed in the level 2 biosecurity microbiology laboratory. The tumour necrosis factor- α (TNF- α) concentration in the milk samples was determined using an enzyme-linked immunosorbent assay (ELISA) kit provided by the Vaccine and Infectious Disease Organization (Saskatoon, Saskatchewan, Canada). The assay was performed according to

the protocol developed at the Vaccine and Infectious Disease Organization and as described previously by other authors (LESSARD *et al.*, 2004).

Fluorescence-activated cell sorting

Measurement of differential leukocyte populations in milk was performed by flow cytometry using the SYTO 13 labelling method, as described previously by other authors (DOSOGNE *et al.*, 2003) but with some modifications. Briefly, 15 mL of collected milk was added to 35 mL of 1X Hank's balanced salt solution without Ca^{2+} or Mg^{2+} (HBSS 10X; Invitrogen, Toronto, Ontario, Canada) and centrifuged. The pellet was re-suspended in RPMI-1640 (Invitrogen) supplemented with 5% fetal bovine serum (FBS; Invitrogen) at 1×10^5 to 1×10^6 cells/mL. Then, 490 μL was transferred to a new tube, and 10 μL of diluted SYTO 13 green fluorescent nucleic acid stain solution (1:400 in RPMI-1640; 5 mM, Invitrogen) was added. After 10 min in the dark, the staining was stopped with 4 mL of HBSS 1X, and the cells were recolled. The pellet was re-suspended in 400 μL of cold RPMI-1640 supplemented with 5% FBS, and was analyzed with a Coulter Epics XL-MCL flow cytometer using Expo 32 software (Beckman Coulter, Mississauga, Ontario, Canada). The forward scatter and side scatter were measured on a linear scale, whereas green fluorescence was registered on a log scale. The differential leukocyte count in milk after SYTO 13 staining was quantified using the side scatter/green fluorescence dot plot. The percentage of different leukocyte populations in milk samples was established, after counting 10,000 events, as follows: percentage of the number of cells in the gated leukocyte population out of the total number of gated lymphocytes, macrophages and granulocytes.

RNA extraction and analysis

Mammary quarters were sampled aseptically. Twenty mL was diluted in an equal volume of PBS and centrifuged at $180 \times g$ for 10 min at 4°C. The cell pellet was washed with cold PBS and 1 mL of TRIzol (Invitrogen, Carlsbad, California, USA) was added. The RNA extraction was performed as recommended by the manufacturer but with minor modifications. The RNA samples were resuspended in water with SUPERase•In (1 U/ μ L; Ambion) and treated with Recombinant DNase I (Ambion). The final concentration was determined using a NanoDrop ND-1000 spectrophotometer. For each cow, equal amounts of RNA from the infected rear quarters and the non-infected forequarters were pooled, with one pool for the infected quarters and one for the non-infected quarters. RNA was amplified using the SMART mRNA Amplification Kit (Clontech, Mountain View, California, USA) according to the manufacturer's protocol. This kit uses the template-switch mechanism to generate the double-stranded cDNA necessary for *in vitro* transcription without the polymerase chain reaction (PCR) step that is generally associated with a conventional SMART technique. The cDNA library was performed according to the recommended protocol for the PCR-Select cDNA Subtraction Kit (Clontech), which is a suppressive subtractive hybridization (SSH) technique. Briefly, equal amounts of amplified RNA from both infected quarters of cows were pooled prior to the hybridizations. The same pooling step was performed for the non-infected quarters. The pool of infected samples was used as the "tester" in accordance with the SSH procedures and was subtracted with an excess of the "driver," the "driver" being the pool prepared from transcripts derived from the non-infected quarters. Consequently, this scheme allows the generation of a differential representation of infected transcripts

normalized with non-infected transcripts. Supplementary PCR amplifications of the hybridization product were then cloned into a TA vector (pCRII, Invitrogen) and transformed into MAX Efficiency DH5 α -competent cells (Invitrogen) to make up a cDNA library. To identify clones containing a single insert, each clone was submitted to PCR using NP1 and NP2R primers, and the products were electrophoresed on a 2% agarose gel. Clones were sequenced according to the BigDye Terminator v3.1 Cycle Sequencing Kit protocol with a 3100-Avant genetic analyzer (Applied Biosystems, Foster City, California, USA). Lastly, gene similarity was searched among our sequences using the Basic Local Alignment Search Tool (BLAST) algorithm of the National Center for Biotechnology Information (NCBI) public database.

Isolation of bovine PBMC and induction

Peripheral blood mononuclear cells (PBMC) were isolated from the buffy coat fractions of peripheral blood and further purified by Ficoll and sucrose gradients. Blood was collected from the jugular or caudal vein in 10 \times heparin (1:10) tubes ($n = 3$ cows). Centrifugation was performed at 572 \times g for 15 min at 18°C. For the respective cow, the fractions were pooled together, completed to 40 mL with 1X HBSS (Hanks' Balanced Salt Solution without Ca²⁺ and Mg²⁺; Wisent, St-Bruno, Canada), poured very slowly onto 2 \times Ficoll gradients (Ficoll Paque Plus; Amersham, Baie d'Urfe, Canada), and centrifuged at 572 \times g for 40 min at 18°C. The PBMC were rinsed with 1X HBSS, re-suspended in 5 mL of 1X HBSS and poured onto a 20% sucrose (Sigma, Oakville, Ontario) discontinuous gradient prior to centrifugation at the same parameters described above. The PBMC were rinsed with 1X HBSS and the cell pellets were treated with Red

Cell Lysis buffer (Sigma) to eliminate residual erythrocytes. Finally, PBMC were re-suspended in 2 mL RPMI-1640 (Wisent) with 5% foetal calf serum (Wisent). Cells were cultured at 5×10^6 in 12 wells flat bottom plate at 39°C in 5% CO₂ in a humidified atmosphere. Cells were incubated for 45 min to allow monocytes to attach before bacterial induction and incubated with either medium alone (nonstimulated) or with heat-inactivated bacteria (63°C for 30 min, as described before (WELLNITZ *et al.*, 2006)) at a concentration of 30 CFU/PBMC. The samples were harvested immediately after the attachment period (time zero) or after 0.5, 1, 3 or 5 h post-infection. Cells in suspension were harvested, 1 mL of TRIzol was added to each well and transferred to the respective pellet cells in order to recover both attached and cells in suspension. The RNA extraction was performed as described above.

RT-PCR and quantitative RT-PCR

The reverse transcription (RT) was performed with 1 µg of RNA according to the SuperScript II RT procedure (Invitrogen). Each RT assay was made in a 20-µl reaction using Oligo(dT)₁₂₋₁₈ (Invitrogen) as a primer and according to the supplier's recommendations. Quantitative RT-PCR (qRT-PCR) was performed in a 10-µL final volume using 5 µL of Fast SYBR Green Master Mix 2X (Applied Biosystems), 300 nM of both forward and reverse primers (Additional table 2), and 2 µL of diluted template. All RT reactions were performed in triplicate. The amplification was carried in a 7500 Fast Real-Time PCR System (Applied Biosystems) following denaturation of 20 s at 95°C and amplification during 40 cycles of denaturation at 95°C for 3 s followed by an annealing/elongation period of 20 s at 60°C. Three reference genes were also measured,

namely actin beta (*ACTB*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and peptidylprolyl isomerase A (*PPIA*). The qRT-PCR results were analyzed according to the relative quantification method given by the arithmetic formula $2^{-\Delta\Delta Ct}$ (LIVAK et SCHMITTGEN, 2001).

Genetic DNA material and EBVs for the SCS and milk production traits

Selection of Holstein genetics was performed using information downloaded from the Canadian Dairy Network database (Guelph, Ontario, Canada), which can also be accessed using the web interface (<http://www.cdn.ca/>). The EBV data for the August 2008 genetic evaluation were downloaded onto a local server. These EBVs comprised the SCS, the non-return rate, and production (protein and fat yields, protein and fat percentages, and milk yield) over lactations. The semen of 578 bulls presenting a mean EBV of 3.01 (Table 8) for the SCS, which is very close to the breed average (3.00; Canadian Dairy Network database), was available for the study. Table 8 describes the EBVs (SCS) for the different lactations. Among the 6,453 recorded bulls, two selections were made using the 150 lowest-ranked and 150 highest-ranked bulls for the EBV for SCS. Among these two groups, semen was available for 51 and 50 bulls, corresponding respectively to the low and high cohorts for the EBV (SCS), and thus defined as the low SCS (2.48 ± 0.07) and the high SCS (3.72 ± 0.17) pools. Genomic DNA (gDNA) was extracted from semen samples, as described previously by other authors (NADESALINGAM *et al.*, 2001) and concentration was measured using the NanoDrop ND-1000 spectrophotometer.

Table 8. Descriptive statistics of the EBVs for the SCS trait for the Holstein bulls^a.

EBV ^b units	Bulls' cohort				Bulls selected for SNP mining							
					Low EBV for SCS				High EBV for SCS			
	Nb	Mean	± SD	[min-max]	Nb	Mean	± SD	[min-max]	Nb	mean	± SD	[min-max]
SCS	578	3.01	± 0.35	[2.34 - 4.16]	51	2.48	± 0.07	[2.34 - 2.72]	50	3.72	± 0.17	[2.99 - 4.16]
SCS1	521	3.00	± 0.31	[2.36 - 4.36]	49	2.53	± 0.08	[2.36 - 2.65]	45	3.63	± 0.18	[3.37 - 4.36]
SCS2	521	3.00	± 0.37	[2.28 - 4.12]	49	2.47	± 0.09	[2.29 - 2.62]	45	3.76	± 0.15	[3.40 - 4.12]
SCS3	521	3.03	± 0.41	[2.08 - 4.15]	49	2.42	± 0.13	[2.13 - 2.70]	45	3.82	± 0.19	[3.42 - 4.26]
Breed average: 3.00					Selected sub-group average: 3.09							

^a EBVs from the Canadian Dairy Network database, evaluation published on August 2008

^b EBVs are calculated for the respective lactation: over all (SCS), first (SCS1), second (SCS2), and third (SCS3) lactations.

SNP mining and sequencing

A sequence of 12,300 bp (GenBank accession No. AY878328) was retrieved by a BLAST search for similarity to a consensus sequence assembled from the *SPP1* transcript sequences from our cDNA library. We used the multiple sequence alignment methods (CLUSTALW) of the DNASTAR suite program MegAlign (Lasergene, v7.2; DNASTAR, Madison, Wisconsin, USA) (data not shown). The sequence of the 5' region of *SPP1* was analyzed *in silico* for identification of transcription factors' recognized DNA binding motifs (Figure 2) using the software MOTIF Search (<http://motif.genome.jp/>) with a cut off score of 85. To construct both pools (high and low EBV for SC) for SNP detection, equal amount of each bulls was PCR-amplified for the respective delimited genomic sequence of *SPP1* (promoter or respective exons) using the appropriate primers designed from the AY878328 sequence (Additional table 3). PCR reactions were carried out in a final volume of 50 μ L containing 2 ng of template, 200 nM of each primer, 200 μ M of each dNTP, 1.5 mM of $MgCl_2$, 1X PCR buffer (20 mM of Tris-HCl pH 8.4, 20 mM of KCl) and 1 U of Taq DNA polymerase (BioShop Canada, Burlington, Ontario, Canada). The conditions were 94°C for 3 min, followed by 35 cycles of 30 s of denaturation (94°C), 30 s of annealing (59°C) and 1 min of elongation (72°C), followed by 5 min of final elongation (72°C). The amplicon size was confirmed by 1.2% agarose gel electrophoresis. The amplified fragments were purified using NucleoFast PCR plates (Macherey-Nagel distributed by MJS BioLynx, Brockville, Ontario, Canada), quantified with the NanoDrop and diluted to a final concentration of 10 ng/ μ L. To construct the selective DNA pools, equal amounts of the amplified genomic fragments from each bull were assembled into the respective high and low EBV (SCS) pools. Two pools

comprising different individuals were assembled, one each for the high and low EBV (SCS) DNA. These pools were used as templates for sequencing with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the company's recommendations, using the forward or reverse PCR primer (Additional table 3) to sequence both strands. When a SNP was detected (overlap of nucleotide bases), all bulls from the pool were individually sequenced to confirm the DNA polymorphisms.

SNP genotyping

Five hundred seventy-eight bulls, from 26 different sires, were genotyped using tetra-primer amplification refractory mutation system PCR (ARMS-PCR), as described previously by other authors (LEYVA-BACA *et al.*, 2008; RINCÓN et MEDRANO, 2003; YE *et al.*, 2001). Primers designed using the open source Cedar Genetics Software (http://cedar.genetics.soton.ac.uk/public_html/primer1.html) are listed in Additional table 4. Reactions were carried out in a 25- μ L final volume containing 50 ng of template in the presence of the appropriate concentration of primers (Additional table 4), 200 μ M of each dNTP, 1.5 mM of $MgCl_2$, 1X PCR buffer (20 mM of Tris-HCl pH 8.4 and 20 mM of KCl), and 1 U of Taq DNA polymerase (BioShop Canada). The PCR reactions were performed at 94°C for 3 min, followed by 35 cycles of 30 s of denaturation (94°C), 30 s of annealing at the appropriate temperature (Additional table 4) and 1 min of elongation (72°C), followed by 5 min of final elongation (72°C). The genotypes were determined according to the fragment length analyzed following 2% agarose gel electrophoresis, as described previously by other authors (LEYVA-BACA *et al.*, 2008).

Statistical analysis

Descriptive statistics for the selective pools (high and low EBV pools for SCS) and the genotyped cohort of 578 bulls are presented in Table 8. Allele and genotype association analyses were done through comparison of the frequencies of the different genotypes and alleles with EBVs for SCS for the respective lactation using logistic regression. Conformance of the allele frequencies with the Hardy-Weinberg equilibrium for all the SNPs was tested using the Chi-square test. All analyses were performed using Statistical Analysis System (SAS) software (release 9.1; SAS Institute, Cary, North Carolina, USA).

The average allele substitution effects of the SNPs were calculated using the model described above (LEYVA-BACA *et al.*, 2008), as follows:

where Y_{jk} is the trait EBV of the j^{th} animal in the k^{th} sire, μ is the overall mean, β_i is the fixed linear regression coefficient for the i^{th} SNP, G_i is the genotype of the i^{th} SNP recoded (ZENG *et al.*, 2005) (namely -1 [homozygous dominant/abundant genotype], 0 [heterozygous genotype] or 1 [homozygous recessive genotype]), S_j is the random polygenic effect of the j^{th} (1 to 26) sire, and e_{jk} is the random error. The bull's EBVs for SCS in the first (SCS1), second (SCS2), third (SCS3) and global (SCS) lactations were used.

Haplotype

The four SNPs detected in the osteopontin gene were used for the construction of haplotype blocks. A total of 578 bulls distributed into 26 Holstein families were selected, thus eliminating families with fewer than 10 offspring. The haplotype reconstruction was performed using the HAPROB algorithm (BOETTCHER *et al.*, 2004), and the results were compared using Haploview (v4.1; (BARRETT *et al.*, 2005)). Each family comprised a minimum of 10 half-sib sons, which is considered a minimum family size for reaching greater than 80% accuracy, as reported previously by other authors (BOETTCHER *et al.*, 2004). From the 578 bulls genotyped, 16 haplotypes were reconstructed, but 11 of those had very low probabilities and were then pooled together to represent a single haplotype for the study on haplotype effect. The linear effects of the six haplotypes were estimated, restricting block H6 to an estimated effect equal to 0 to account for a linear dependency among haplotype effects, as described elsewhere (LEYVA-BACA *et al.*, 2008). Haplotype effects were estimated with PROC MIXED of SAS (SAS Institute), using the same model as described above (LEYVA-BACA *et al.*, 2008), as follows:

where Y_{jk} is the trait EBV of the j^{th} animal in the k^{th} sire, μ is the overall mean, β_i is the fixed linear regression coefficient for the i^{th} haplotype, Hap_{ik} is the probability of the i^{th} haplotype for the k^{th} bull, S_j is the random polygenic effect of the j^{th} (1 to 26) sire, and e_{ijk} is the random error. Results were considered significant if P values were less than 0.05.

AUTHORS' CONTRIBUTIONS

CT and JS participated in the design and carried out the experimentation on the cows (bacterial challenge and sample collection). JS performed the bacterial count, KA and CT performed the molecular experiments. ML's team performed the fluorescence-activated cell sorting assays. NAK's team provided 500 bull DNA samples. NB conceived the study. KA and NB interpreted the results and wrote the manuscript. NAK, ML and CT revised the manuscript. All authors read and approved the final manuscript.

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ADDITIONAL FILES

Additional Figure 1. Sequence alignment of the different regions of *SPP1* surrounding (A) -2,419 nucleotides (nt) (T₉/T₁₀ INDEL), (B) -1,301 nt (*SPP1c*.-1301G>A), and (C) -1,251 nt (*SPP1c*.-1251C>T).

^aComplement of nt 3,878–3,916¹, nt 5,015–5,035² and nt 5,065–5,085³ of GenBank accession No. AY878328.

^bComplement of nt 14,313,006–14,313,044¹, nt 14,314,352–14,314,372² and nt 14,314,403–14,314,424³ of GenBank accession No. NC_006614.

^cComplement of nt 1,130–1,166¹, nt 2,148–2,168² and nt 2,199–2,220³ of GenBank accession No. D14813.

^dComplement of nt 90,886,781–90,886,817¹, nt 90,887,798–90,887,818² and nt 90,887,849–90,887,870³ of GenBank accession No. NC_006471.

^eComplement of nt 1,660–1,698¹, nt 3,039–2,059² and nt 2,092–2,113³ of GenBank accession No. D14816.

^fComplement of nt 3,339–3,374¹, nt 2,495–2,515² and nt 2,544–2,564³ of GenBank accession No. M84121.

Additional Table 1. Effects of polymorphisms in the bovine *SPP1* gene on EBVs for production traits.

DNA polymorphism	Genotype	Milk yield (Kg)	Fat yield (Kg)	Protein yield (Kg)	Fat %	Protein %
Least squares mean						
<i>SPP1c.-1301G>A</i>	GG	-67	-2	-2	0.01	0.02
	GA	-134	1	< -1	0.06	0.04
	AA	-517	-11	-10	0.01	0.07
	± SEM	± 328	± 11	± 9	± 0.12	± 0.05
	<i>p</i> value	0.304	0.410	0.602	0.288	0.074
<i>SPP1c.-1251C>T</i>	CC	-36	-1 ^a	< 1	0.01 ^{a,b}	0.02
	CT	-152	< -1 ^a	-2	0.06 ^a	0.04
	TT	-99	-13 ^b	-2	-0.09 ^b	0.02
	± SEM	± 132	± 4	± 4	± 0.05	± 0.02
	<i>p</i> value	0.257	0.026	0.630	0.012	0.169
<i>SPP1c.-430G>A</i>	GG	-36	-1 ^a	< 1	0.01 ^{a,b}	0.02
	GA	-152	< -1 ^a	-2	0.06 ^a	0.04
	AA	-99	-13 ^b	-2	-0.09 ^b	0.02
	± SEM	± 132	± 4	± 4	± 0.05	± 0.02
	<i>p</i> value	0.257	0.026	0.630	0.012	0.169
<i>SPP1c.*40A>C</i>	AA	-17	-1 ^a	1	< .01	0.02
	AC	-144	< -1 ^a	-1	0.06	0.04
	CC	-176	-11 ^b	-4	-0.04	0.02
	± SEM	± 111	± 4	± 3	± 0.04	± 0.02
	<i>p</i> value	0.135	0.022	0.352	0.031	0.110

^{a,b,c} Means (within a line) without a common superscript letter differ from each other at

the 5% level of significance.

Additional Table 2. Oligonucleotide primer sequences for quantitative real-time PCR.

Gene name		Primer sequences (5'-3')	Size bp
<i>SPP1</i> ^a	Forward	GCTTCATGGTAACACCCTTCTAAACT	137
	Reverse	TGTTTGCTTCTACATGAATTTCTATTCC	
<i>TNF</i> ^b	Forward	GACGGGCTTTACCTCATCTACTCA	100
	Reverse	AGACTGCAATGCGGCTGAT	
<i>ACTB</i> ^c	Forward	TGGCACCCAGCACAAATGA	123
	Reverse	CCTGCTTGCTGATCCACATCT	
<i>GAPDH</i> ^d	Forward	CAGGGTGGTGGACCTCATG	138
	Reverse	GGAGATTCTCAGTGTGGCGG	
<i>PPIA</i> ^e	Forward	ATGCTGGCCCCAACACAA	101
	Reverse	CCCTCTTTCACCTTGCCAAA	

^a Complement of nt 1064 to 1200 of GenBank accession no. NM_174187

^b Complement of nt 582 to 681 of GenBank accession no. NM_173966

^c Complement of nt 1051 to 1173 of GenBank accession no. NM_173979

^d Complement of nt 1032 to 1169 of GenBank accession no. NM_001034034

^e Complement of nt 317 to 417 of GenBank accession no. NM_178320

Additional Table 3. Sequencing primers for *SPPI*.

Genomic regions		Primer sequences (5'-3') For Amplification and Sequencing	Annealing °C	Size bp
Promoter ^a	Forward	TGGTGCCAAAGAAAAGTT	59	1033
	Reverse	GTGCAGCAGCTTGTATGTAGAC		
	Seq. ^h	TCTGTCAAATGCAGAGTTCGTGGG		
Promoter + Exon 1 ^b	Forward	AGTTATCAGATCCATCAGCTCCAAA	59	850
	Reverse	CTTACAAATTGACCTTCCCAATGA		
Intron 1 ^c	Forward	ATTCTGGGAGATCCTGGTTGTCAG	59	1408
	Reverse	GCCAGAACTGGTCGGTTTAACCTTTG		
	Seq. ^h	CCTTGACTTACTAACCTTAGAGACAGC		
	Seq. ^h	ACTATTAGGCAAGGGAGAGAGG		
Exons 2-3 (+intron2) ^d	Forward	GAGATGGAAAATAGAGGTGGCAGTA	59	545
	Reverse	AAGCATAGCAGGCACACAATAAAT		
Exons 4-5 (+intron4) ^e	Forward	TTGTCAAGATTGGAGAAGAACAGTTG	59	809
	Reverse	CGGAATTTGAACCTGGGCAATGTC		
Exon 6 ^f	Forward	TGCACTGTAAAGCCTAAGGGACAG	59	436
	Reverse	GCAGACTCTAGTTTCCTAGAATTGGGC		
Exon 7 ^g	Forward	TTACAGTGCTTCCCTTCCTAGCTG	59	974
	Reverse	ACGGGAACCTTTAGCTCAGTCAGAGTC		

^a Complement of nt 3561 to 4593 of GenBank accession no. AY878328.

^b Complement of nt 4448 to 5297 of GenBank accession no. AY878328.

^c Complement of nt 5111 to 6518 of GenBank accession no. AY878328.

^d Complement of nt 6123 to 6667 of GenBank accession no. AY878328.

^e Complement of nt 8894 to 9702 of GenBank accession no. AY878328.

^f Complement of nt 10315 to 10750 of GenBank accession no. AY878328.

^g Complement of nt 11322 to 12295 of GenBank accession no. AY878328.

^h Primers only used for sequencing (Seq.).

Additional Table 4. Primers for SNP genotyping using tetra-primer ARMS-PCR in the bovine *SPP1* gene.

Polymorphism	Primer Sequence (5'-3')	Primer final [nM]	Annealing °C	Amplicon bp
<i>SPP1c.-1301G>A</i>				
Inner Forward	CCACAAAACCAGAGGGGGAAGTGTGGT <u>I</u> AA	100	61	244 (G)
Inner Reverse	ATTTTGCCACTACCCAGCCCACT <u>G</u> GC	100		193 (A)
Outer Forward	AACCCTTTTCCCTCCCTCTACGTTTTCATGG	10		382
Outer Reverse	CTGTGATGCTGATGCAGTGCTCAGTGCT	10		382
<i>SPP1c.-1251C>T</i>				
Inner Forward	AAATGCCCCATGACACATCTCTCCGC <u>A</u> CT	25	65	269 (C)
Inner Reverse	TGCTCCAGATGCTCTCCACCTACAC <u>C</u> GG	50		223 (T)
Outer Forward	ATGGGCCCTCTAGATGCCCTTCCAGGATG	10		435
Outer Reverse	GGCAGCGCACAGAGAGGACAGGAGTCTG	10		435
<i>SPP1c.-430G>A</i>				
Inner Forward	AAATGCATGTTGGAAAATGGAGAA <u>A</u> AA	300	59	271 (G)
Inner Reverse	GAAGATAAAAATAAGAAAGAGAAACT <u>G</u> GC	200		177 (A)
Outer Forward	AAAAAAACCCTTTTCTGAATATTTTCACC	75		393
Outer Reverse	ACACCATTACTACCTGATATCTCCAGCT	50		393
<i>SPP1c.*40A>C</i>				
Inner Forward	ACAATTTCTTACTTTTGCTTTT <u>A</u> GTAA <u>G</u> AC	200	59	145 (A)
Inner Reverse	CACCCTGCTTTAATGTATCCTTT <u>C</u> CT	200		197 (C)
Outer Forward	ATCATAAGAGTGAAGAAGACAAACACT	50		287
Outer Reverse	AAACTCTGAAGCTTTTAGTTT <u>A</u> GAA <u>G</u> GG	50		287

^a Nucleotide underlined corresponds to a mismatch introduced according to the tetra-primer ARMS-PCR assay to increase annealing specificity to perfect nucleotide match.

DISCUSSION ET CONCLUSION

La technique de normalisation des transcrits utilisée dans cette étude s'est avérée très efficace pour l'étude des gènes de l'immunité innée sur des cellules du lait recueillies avant même les premiers signes d'infection occasionnés par une mammite de type clinique. Le transcriptome des cellules prélevées lors de l'infection présentait un niveau d'expression du gène *SPP1* plus élevé que le niveau détecté avant le début de l'infection. La population d'ADNc a effectivement été enrichie en transcrits de l'ostéopontine différenciellement exprimés grâce à cette méthode d'hybridation suppressive soustractive (DIATCHENKO *et al.*, 1996). La détection de *SPP1* précède même *TNF α* , un médiateur inflammatoire important impliqué dans le recrutement et l'accumulation des neutrophiles à la glande mammaire lors d'infection bactérienne (CYBULSKY *et al.*, 1988; RIOLLET *et al.*, 2000). Pour ces raisons, l'ostéopontine devient un candidat intéressant, d'autant plus qu'il est reconnu comme étant un modulateur important du fonctionnement efficace de toute réponse immunitaire innée. Des recherches plus approfondies seront nécessaires afin de mieux comprendre ses rôles dans la mammite.

Grâce au séquençage de taureaux présentant des cotes extrêmes pour les cellules somatiques, quatre polymorphismes d'un seul nucléotide furent trouvés. Trois SNP, *SPP1c.-1251C>T* et *SPP1c.-430G>A* en 5' UTR et *SPP1c.*40A>C* en 3' UTR, avaient déjà été découverts dans une étude antérieure (SCHNABEL *et al.*, 2005). Cependant, la technique que nous avons privilégiée s'est avérée très efficace et a permis la découverte d'un nouveau SNP (*SPP1c.-1301G>A*) dans la région promotrice du gène, en amont des autres polymorphismes de l'ADN. Les analyses statistiques réalisées (moindre carré

moyen, effet moyen de substitution allélique, effet estimé d'haplotype) en utilisant l'information reliée aux différents polymorphismes de l'ADN et haplotypes de *SPP1* confirment qu'il y a bel et bien un lien avec la quantité de cellules somatiques, et fort probablement un lien avec la mammite, un lien qui demeure cependant à être validé.

Suivant l'analyse *in silico* de la région promotrice de l'ostéopontine bovine, plusieurs motifs putatifs de liaison de facteurs de transcription à l'ADN furent révélés. Afin de mettre en lumière le rôle fonctionnel de ces polymorphismes de l'ADN, soit leur influence sur la reconnaissance de facteur de transcription ayant un impact sur l'immunité de la glande mammaire, l'étude d'association entre les allèles et les valeurs phénotypiques furent démontrées. Pour le polymorphisme situé à 1301 nucléotides en amont du codon d'initiation de la traduction (ATG), l'allèle G comporte un site SP1 (facteur de transcription Sp1) à proximité (à environ 5 nucléotides, voir Figure 2). Pour l'allèle A, en plus du motif SP1, on voit apparaître un site IKZF2 (Ikaros family zinc finger 2) (Figure 2). La région contenant ce SNP semble bien conservée entre les différentes espèces (bovin, canin, humain, chimpanzé et porc) sauf peut-être pour la souris (Figure supplémentaire 1 B). L'allèle C du polymorphisme de nucléotide simple situé à -1251 nucléotides de l'ATG comporte également un site SP1 qui est aboli en présence de l'allèle T (Figure 2). Encore une fois, cette région du promoteur est très conservée entre les différentes espèces comparées (Figure supplémentaire 1 C). Le site SP1 se trouvant à proximité du polymorphisme *SPP1c.-1251C>T* a d'abord été retrouvé chez l'humain (HUMMELSHOJ *et al.*, 2006). Les auteurs de cet article observent, entre autres, qu'en substituant un allèle pour un autre, on voit un effet sur l'activité promotrice et une

influence sur l'équilibre entre les réponses immunitaires de type Th1 et Th2. Le facteur de transcription SP1 favoriserait l'activité Th1; les individus ayant les allèles de *SPP1* liant Sp1 auraient probablement une meilleure stimulation des cellules phagocytaires et une destruction plus efficace des pathogènes intracellulaires par rapport à une immunité de type Th2. L'allèle C du polymorphisme d'un seul nucléotide *SPP1c.-1251C>T* diminue effectivement la VÉE pour le CCS par rapport à l'allèle G, ce qui nous permet d'émettre l'hypothèse suivant laquelle, grâce au site SP1, cet allèle procure une défense accrue contre la mammite via une immunité cellulaire favorisée et une activité phagocytaire plus efficace contre les bactéries. Le facteur de transcription IKAROS, quant à lui, exerce plutôt l'action contraire, c'est-à-dire qu'il favoriserait la voie Th2, comme on l'a récemment rapporté (QUIRION *et al.*, 2009). En effet, les auteurs ont démontré que les souris *Ikaros*^{-/-} sécrètent peu de cytokines associées à la voie de l'immunité de type Th2. Cela nous amène à poser l'hypothèse selon laquelle le site IKZF2 rendu accessible sur l'allèle A par la présence du SNP *SPP1c.-1301G>A* de l'ostéopontine permettrait la liaison du facteur IKAROS qui aurait pour conséquence d'inhiber la transcription de cette cytokine de type Th1 pour favoriser la voie Th2. Ainsi, on observerait une moins bonne réponse immunitaire innée et par conséquent, une susceptibilité aux bactéries intracellulaires augmentée (la voie Th1 favorise l'activité des cellules phagocytaires). Par ailleurs, suivant la liaison du facteur IKAROS, la liaison de SP1 situé à proximité de l'allèle A pourrait être compromise. C'est ce qui expliquerait le fait que l'allèle A augmente la VÉE pour le CCS (risque plus élevé de mammite) par rapport à l'allèle G, où le site SP1 ne serait pas sous l'influence du site de reconnaissance adjacent. À proximité du polymorphisme *SPP1c.-430G>A* se trouve deux cibles putatives pour les sites de

liaison au facteur nucléaire des lymphocytes T activés (NFAT) et à l'élément de réponse à la stimulation par l'interféron (ISRE), deux molécules reconnues pour réguler l'expression de *SPP1* (LI *et al.*, 2003; NILSSON *et al.*, 2006; ZETTERQVIST *et al.*, 2008). Comme l'allèle A diminue la VÉE pour le CCS, ce polymorphisme favoriserait la liaison de ces facteurs de transcription et l'expression de *SPP1* serait plus élevée par rapport à l'allèle G. Une meilleure expression de *SPP1* stimulerait davantage l'immunité innée et la défense contre les agents responsables de la mammite. Finalement, le dernier polymorphisme *SPP1c.*40A>C* se trouve en 3' UTR. Cette région de l'ARNm est connue pour être la cible de microARN (MAJOROS et OHLER, 2007). Ces petits ARN se lient à des sites spécifiques de l'ARNm et suppriment l'expression des protéines, en favorisant la dégradation du transcrit ou l'inhibition de la traduction par exemple (MAJOROS et OHLER, 2007). Il se pourrait donc que le polymorphisme *SPP1c.*40A>C* soit situé dans un site de liaison pour les microARN et puisse abolir la liaison de microARN à l'allèle C. Ceci stabiliserait les ARNm et augmenterait la réponse Th1 puisqu'on observe une diminution de la VÉE de la CCS pour les animaux comportant cet allèle. Ainsi, l'expression de *SPP1*, plus soutenue avec l'allèle C comparé à l'allèle A, favoriserait une meilleure défense contre les infections bactériennes. Ces hypothèses méritent d'être vérifiées afin de valider l'effet de la présence de ces polymorphismes de l'ADN (et haplotypes) de *SPP1* sur l'expression du gène dans des conditions qui confèrent réellement une meilleure immunité face aux pathogènes responsables de la mammite.

Il est possible de vérifier la présence de certains éléments de régulation génétique aux sites d'emplacement des polymorphismes de l'ADN identifiés. On pourrait donc détecter la liaison (ou non) des facteurs de transcription mentionnés ci-dessus aux différentes séquences promotrices. Ainsi, nous pourrions confirmer l'influence des polymorphismes d'un seul nucléotide sur la capacité de liaison des facteurs de transcription. Certaines méthodes moléculaires permettent l'analyse des interactions entre protéines et ADN. En l'occurrence la technique connue sous le nom LMPCR pour « ligation-mediated polymerase chain reaction » (DROUIN *et al.*, 2009) le fait de façon *in cellulo*, ce qui est encore plus intéressant que la technique de l'immunoprécipitation de la chromatine (CHIP). Pour ce qui est des microARN, il existe des méthodes informatiques pour prédire les séquences cibles d'un microARN sur l'ARNm d'un gène (e.g. programme de prédiction miRanda, <http://www.microrna.com>), cependant ces algorithmes sont conçus pour l'humain et la souris. Afin de valider l'effet des différents SNP sur l'activité promotrice de *SPP1*, des essais avec le gène rapporteur de la luciférase (GIACOPELLI *et al.*, 2004; HUMMELSHOJ *et al.*, 2006) seront éventuellement réalisés et permettront de savoir quels SNP inhibent ou favorisent l'activité du gène. Des essais d'infection *ex vivo* sur du lait ou des cellules mononuclées du sang périphérique (PBMC) de vaches saines présentant différents haplotypes pour *SPP1* seront aussi entrepris. L'expression du gène *SPP1* pourra ensuite être quantifiée par qPCR et comparée entre les différents haplotypes. Ainsi, nous serons plus en mesure de confirmer quels SNP et haplotypes favorisent l'expression de *SPP1* et augmentent les défenses naturelles des animaux face à la mammite. Ces allèles favorables pourront éventuellement être ciblées et servir de

marqueurs pour la sélection génétique de taureaux géniteurs pour améliorer la longévité du troupeau laitier.

En conclusion, le gène *SPP1* de l'ostéopontine est surexprimé lors de cas de mammite. L'ADN génomique de cette cytokine contient des variations génétiques et les variants alléliques ont un lien établi avec le compte de cellules somatiques. On observe d'ailleurs des fréquences alléliques différentes lorsqu'on compare les populations de taureaux avec des valeurs de CCS extrêmes. Certaines variations génétiques de l'ADN de l'ostéopontine aboliraient des sites de liaisons de protéines importantes et ainsi, les différents variants ne semblent pas offrir la même efficacité des réponses immunitaires de type Th1 et Th2. On aurait donc intérêt à sélectionner des taureaux géniteurs qui comportent les allèles favorables afin d'augmenter la fréquence des polymorphismes bénéfiques dans la descendance et lui inférer une meilleure résistance suivant une insulte par des pathogènes causant la mammite.

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